

P A T COOPERATION TREATY

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To

Invention Office of the Democratic
People's
Republic of Korea
Kinmaul 1 dong, Bipa Street,
Moranbong District
Pyongyang
RÉPUBLIQUE POPULAIRE DÉMOCRATIQUE
DE CORÉE

in its capacity as elected Office

Date of mailing (day month year) 08 March 2000 (08.03.00)	
International application No. PCT/US99/17177	Applicant's or agent's file reference N1121-037.PCT
International filing date (day/month/year) 29 July 1999 (29.07.99)	Priority date (day/month/year) 31 July 1998 (31.07.98)
Applicant GOLDSMITH SEEDS, INC.	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:

15 February 2000 (15.02.00)

☐ in a notice effecting later election filed with the International Bureau on:2. The election ☒ was☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To

Assistant Commissioner for Patents
 United States Patent and Trademark
 Office
 Box PCT
 Washington, D.C. 20231
 ÉTATS UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day month year) 08 March 2000 (08.03.00)	
International application No. PCT/US99/17177	Applicant's or agent's file reference N1121-037.PCT
International filing date (day month year) 29 July 1999 (29.07.99)	Priority date (day month year) 31 July 1998 (31.07.98)
Applicant BOWMAN, Robert, N.	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:
 15 February 2000 (15.02.00)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was
☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

REC'D 15 SEP 2000

WIPO PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference N1121-037.PCT		FOR FURTHER ACTION		See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US99/17177	International filing date (day/month/year) 29/07/1999	Priority date (day/month/year) 31/07/1998		
International Patent Classification (IPC) or national classification and IPC C07D519/04				
Applicant GOLDSMITH SEEDS, INC. et al.				

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 8 sheets, including this cover sheet.
 - ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☒ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand

Date of completion of this report

International Preliminary Examining Authority



European Patent Office - Gitschiner Str. 103
D-10958 Berlin
Tel. +49 30 25901-0
Fax +49 30 25901-840

Hass, C



**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/US99/17177

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-32 as originally filed

Claims, No.:

1-35 as originally filed

Drawings, sheets:

1/24-24/24 as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

- ☐ the entire international application.

because.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US99/17177

- ☒ the said international application, or the said claims Nos. 26-28,32,33 relate to the following subject matter which does not require an international preliminary examination (*specify*):

see separate sheet

- ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

- ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

- ☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes: Claims 2-35
	No: Claims 1
Inventive step (IS)	Yes: Claims 2-35
	No: Claims 1
Industrial applicability (IA)	Yes: Claims 1-25,29-31,34,35
	No: Claims

2. Citations and explanations

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/US99/17177

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claims 26 to 28, 32 and 33 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Cited documents

D1: Journal Of Medicinal Chemistry (1979), 22(4), 391-400
D2: US-A-4199504
D3: EP-A-233101
D4: US-A-5024835
D5: EP-A-222722
D6: US-A-5030620
D7: EP-A-41935
D8: EP-A-124502
D9: US-A-4203898
D10: US-A-5491285
D11: US-A-4831133
D12: US-A-4172077

The numbering will be adhered to throughout the report.

2. Novelty

Claim 1 comprises any indole alkaloid compound having a molecular weight greater

than 200, and a method of producing the same, wherein the compound is a

the method of its production; consequently, D1 to D9 destroy the novelty of claim

1, since all of them disclose indole alkaloid compounds having a molecular weight greater than 980 (see the relevant passages cited in the international search report).
Claim 2, 4 to 13: No trimeric vindoline-based alkaloid compounds are disclosed in any of the documents D1 to D12. The subject-matter of claim 2 and (consequently) of depending claims 4 to 13 is thus novel.

Claim 3: No compound is disclosed in any of D1 to 12, which is isolated from a *Catharanthus* plant and which has a molecular weight of greater than 980; all compounds disclosed in D1 to D9 which have a molecular weight greater than 980 are chemically modified compounds.

Claims 14, 15, 16 to 19, 20, 21: No extract of a *Catharanthus* plant containing a compound with a molecular weight greater than 980 is disclosed in any of the cited documents. Therefore the subject-matter of the respective claims 14, 15, 16 to 19, 20, 21 is novel.

Claims 22-35 depend on at least one of the claims mentioned above (at least indirectly), the subject-matter of these claims is therefore novel, too.

3. Inventive step

3.1 According to the description, the problem underlying the present application is to provide further indole alkaloids, which can be extracted from certain *Catharanthus* plants and which are useful as medicines for animal (including man) and plant diseases.

3.2 The Applicant has shown that trimeric indole alkaloid compounds are produced by complex *Catharanthus* interspecific hybrids, and these compounds can be obtained by extracting them from these plants. Some compounds have been isolated which are said by the Applicant to be trimeric indole alkaloids (the mass spectra are in accordance with this assumption) and which are said to be more or less biologically active, e.g. as antifungal or antimicrobial, but also as anticancer agents.

3.3 D1 is considered to represent the closest prior art, since this document discloses *Catharanthus* alkaloids having anticancer activity, especially two compounds, namely compounds 32 and 37, having molecular weights greater than 980, and which are said to be more or less biologically active, e.g. as antifungal or antimicrobial, but also as anticancer agents. In the light of D1, the problem which has been solved by the

present application is to provide further biologically active indole alkaloids which contain more than two monomers.

3.4 Concerning the isolation of indole alkaloids from *Catharanthus* plants, D10 to D12 are considered relevant as well. Especially D10 discloses *Catharanthus* plants resistant to *Phytophthora*, which plants are described to contain an increased level of total alkaloid content.

3.5 The person skilled in the art, searching for the solution of the problem defined above (provision of *further* indole alkaloid compounds) would, on the one hand, deduce from D1 that also alkaloids having more than two monomeric units can have biological activity; however, on the other hand, he or she cannot assume that alkaloids having more than two monomeric units could be directly extracted from certain *Catharanthus* varieties. Therefore it can be said that the subject-matter of the present application (as far as it is novel) **in principle** involves inventive step. However, it should have been ensured that the claims only comprise such subject-matter which actually appears to solve the problem underlying the application (breadth of claims in conjunction with inventive step). Moreover, claim 1 have to comprise all measures which are essential to carry out the invention. Therefore the following features should have been included in claim 1:

- An indication that the compounds in question are trimers ("higher" molecules are described by the Applicant to have been found as peaks e.g. in HPLC chromatograms, however, it is not clear that these "polymeric alkaloids" also have biological activity, i.e. that they solve the problem underlying the application - maybe they are merely by-products of the active trimers) and that these trimers are direct extraction products of certain plants;
- a definition of the plants used in order to make it clear to the reader of the claims from which *catharanthus* plants these trimers can be extracted (an essential feature of these plant species appears to be their resistance to *Phytophthora*);
- a molecular weight range which is not "open" (as in present claims 1, 2, 4-6, where only the lower limit is defined, i.e. which is defined by a lower and an upper limit). These restrictions appear to be necessary since the "core" of the invention seems to be represented by the specific plant varieties which used to produce specific biologically

claim 1 cannot be considered inventive since it already discloses tetramers

species which are biologically active (see above).

Re Item VIII

Certain observations on the international application

Clarity (Art. 6 PCT): For the sake of clarity, the term "about" in connection with ranges is to be objected to; this objection applies to claims 1, 2, 3, 14, 18, 19, 20 and 21. In claim 17, the expression "and other monomers" is not clear; moreover, it seems not to be supported by the description, since no monomers other than catharanthine and vindoline explicitly appear to be mentioned in the description.

Re Item VII

Certain defects in the international application

The wording of claim 7 is to be objected to, since this claim relies on references to the description ("Table 5"), which is not acceptable in view of Rule 6.2(a) PCT. Document D1 should have been referred to in the description since it represents relevant prior art (Rule 5.1(a)(ii) PCT).



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷: C07D 519/04, 519/00, C07G 5/00, A61K 31/475	A1	(11) International Publication Number: WO 00/06582 (43) International Publication Date: 10 February 2000 (10.02.00)
(21) International Application Number: PCT/US99/17177 (22) International Filing Date: 29 July 1999 (29.07.99) (30) Priority Data: 60/095,000 31 July 1998 (31.07.98) US (71) Applicant (for all designated States except US): GOLDSMITH SEEDS, INC. [US/US]; 2280 Hecker Pass Highway, Gilroy, CA 95020 (US). (72) Inventor; and (75) Inventor/Applicant (for US only): BOWMAN, Robert, N. [US/US]; 1471 Amber Court, Gilroy, CA 95020 (US). (74) Agents: JONDLE, Robert, J. et al.; Rothwell, Figg, Ernst & Kurz, Suite 701 East, 555 13th Street N.W., Columbia Square, Washington, DC 20004 (US).	(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>	

(54) Title: TRIMERIC AND POLYMERIC ALKALOIDS**(57) Abstract**

The present invention relates to extracts of disease-resistant *Catharanthus* plants, to trimeric and polymeric alkaloids produced by these plants and to the use of the extracts, trimeric alkaloids and polymeric alkaloids as medicinals and anti-biological agents.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BF	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BE	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NI	Netherlands	VN	Viet Nam
CG	Congo	KF	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea				

DK
Denmark

GB
Ireland

SE
Sweden

SG
Singapore

TITLE OF THE INVENTION

TRIMERIC AND POLYMERIC ALKALOIDS

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority from a provisional application which was filed in the
5 United States on July 31, 1998, having Serial No. 60/095,000, incorporated herein by reference.

BACKGROUND OF THE INVENTION

The present invention relates to extracts of disease-resistant *Catharanthus* plants, to
trimeric and polymeric alkaloids produced by these plants and to the use of the extracts, trimeric
10 alkaloids and polymeric alkaloids as medicinals and anti-biological agents.

The publications and other materials used herein to illuminate the background of the
invention, and in particular, cases to provide additional details respecting the practice, are
incorporated by reference, and for convenience are referenced in the following text by author and
date and are listed alphabetically by author in the appended list of references.

15 *Catharanthus roseus* (L.) G. Don, also called periwinkle, originates from Madagascar and
belongs to Apocynaceae family. *Catharanthus* spp. are well known for their production of indole
alkaloids (Farnsworth, 1961; Taylor and Farnsworth, 1975). *Catharanthus* is one of the most
extensively studied medicinal plants. Since 1950, well over 1200 scientific publications, including
about 90 patents dealing with this plant have appeared (Moreno et al., 1995). *Catharanthus roseus*
20 produces a large variety of monomeric indole alkaloids (Lounasmaa and Galambous, 1989). In
addition to the 100 or more known natural monomers, *Catharanthus roseus* also produces dimeric
indole alkaloids including naturally-occurring vinblastine and vincristine, two medically important
anti-tumor agents used in the chemical treatment of human cancers.

As a consequence of the discovery of anti-cancer activity associated with *Catharanthus*
25 alkaloids (Svoboda and Blake, 1975), numerous studies on the pharmacological activity of these
compounds have occurred. Within the monomeric group of alkaloids, none have been shown to
have significant anti-cancer activity, and only two (ajmalicine as an anti-hypertensive agent and
serpentine as a sedative) are of minor commercial value (Moreno et al., 1995). In contrast,
essentially all of the natural dimers appear to have at least some associated anti-cancer activity

research on cell biology of *Catharanthus roseus* for well over 30 years. Despite such rigorous

research effort aimed at cell or tissue culture (DiCosmo and Misawa, 1995) or in vitro synthesis (Kutney, 1990; U.S. Patent No. 5,047,528), massive quantities of whole plant parts (especially leaves) are still the commercial source for these bisindole alkaloids.

Dimeric indole alkaloids in *Catharanthus* are formed by *in vivo* condensation of vindoline and catharanthine monomers. Meijer et al. (1993) and Sottomayor et al. (1997) have reviewed enzymatic aspects of catharanthine and vindoline biosynthesis in *Catharanthus* leading to coupling to form anhydrovinblastine, the precursor of other bisindoles including vinblastine, vincristine, leurosine and catharine. While the monomer catharanthine is found in all parts of *Catharanthus roseus* plants as well as in root or shoot cultures, vindoline and the bisindole alkaloids accumulate only in whole green parts, including shoot cultures. The biosynthesis and accumulation of vindoline in the intact plant is controlled by tissue-specific, developmentally regulated and light-dependent factors (Aerts and DeLuca, 1992; St-Pierre and DeLuca, 1995). Dependence on *in planta* synthesis of vindoline and the general rarity of bisindoles in whole leaves (.003% dry wt.; Sottomayor et al., 1996), contributes to the high cost of the bisindole chemotherapeutics.

All of the known naturally occurring bisindoles, including vinblastine and vincristine, are representable by the formula shown in Figure 1. The upper heterocyclic component is the catharanthine monomer and the lower heterocyclic monomer is the vindoline monomer. In Figure 1, numbering conventions of previous U.S. patents 3,932,417; 4,303,584; 4,199,504; 4,203,898; 4,375,432; 4,479,957 have been followed. In the formula of Figure 1, vinblastine (VB) is represented by R being methyl and vincristine (VC) is represented by R being formyl. It should be noted that other numbering conventions (including IUPAC carbon numbering) are frequently encountered in the literature; variance in numbering nomenclature greatly contributes to confusion in comparisons of alkaloid ring structures. Figure 1 has retained the numbering conventions of early Eli Lilly patents to be consistent with the existing large body of bisindole alkaloid literature. This numbering system will be referred to herein with reference to, e.g., unsaturation or saturation.

Even though the structural difference between vinblastine ($R = CH_3$) and vincristine ($R = CHO$) is minor, the compounds exhibit substantial differences in pharmacological as well as toxicological activity (Bruneton, 1995). VB and VC have significant clinical anti-tumor activity

Based on structure-activity relationships of the naturally occurring dimers, it is evident

that the vindoline-derived moiety of the naturally occurring dimers significantly influences anti-cancer activity of the dimer molecule. For example, it is known that unsaturation at the C6-C7 bond in VB and VC is required for biological activity. If this bond is saturated, anti-cancer activity is significantly eliminated.

5 As a consequence of the significant clinical anti-tumor activity of VB and VC, much research effort has centered on dimer structure and molecular aspects of alkaloid biogenesis in *Catharanthus*. Metabolic aspects of bisindole biosynthesis in *Catharanthus roseus* have been reviewed by Kutney (1990) and Kutchan (1995). Working with *C. roseus*, Kutney (1990) has summarized the major aspects of dimer synthesis leading to structural differences in the naturally
10 occurring dimers. The bisindole 3',4'-anhydrovinblastine (AHVB), a known naturally-occurring precursor to all of the natural dimers (Kutney, 1990; Endo et al., 1988), also possesses significant anti-cancer activity (IGT pharma, 1998). AHVB differs from VB and VC in that the former possesses structural differences in the catharanthine moiety of the dimer molecule.

Beyond their own inherent chemotherapeutic value, the naturally occurring bisindoles from
15 *Catharanthus* provide useful starting points in the *in vitro* synthesis of structurally related analogs and derivatives. Barnett et al. (1978) prepared deacetylvinblastine amide (vindesine, Figure 2) from VB. Phase I and II clinical trial reports (Dyke and Nelson, 1977) indicate vindesine to be an active oncolytic agent. Clinically, vindesine appears to be less toxic than VC while having an activity spectrum similar to VC rather than its parent VB. The structural similarity of vindesine
20 to VC and VB (the former possessing an amide substitution at C3 on the vindoline moiety) further emphasizes the significance of the vindoline moiety in achieving anti-cancer activity. Conrad et al. (1979), in a comprehensive examination of 41 synthetic N-substituted deacetoxyvinblastine amide sulfates (all synthesized from VB C3 substitutions), further demonstrated the importance of the vindoline moiety in expressing anti-cancer activity; thus, "minor" structural differences in
25 VB modification products attributable to substitutions at the C3 position of the vindoline moiety can be related to the experimental anti-tumor response spectrum and toxicological aspects of the molecule.

Over the course of more than 30 years of research covering structural modification of the natural dimer molecules, various structural synthetic analogs have been produced involving

... demonstrated pharmaceutical properties. European Patent 0,010,458 describes the synthesis of navelbine, a

vinblastine derivative with demonstrated anti-cancer activity. U.S. Patent 5,024,835 describes vinblastine derivatives carrying a detergent chain. U.S. Patent 5,030,620 describes vinblastine-related derivatives containing a protein fragment addition. U.S. Patent 3,352,868 describes the synthesis of dihydrovinblastine by low pressure hydrogenation of vinblastine. The above-mentioned synthetic analogs all possess a single catharanthine and vindoline moiety; all exhibit anti-cancer efficacy though as exemplified by dihydrovinblastine, at levels lower than the natural parent compound. High initial cost of the bisindole reactants (VB - \$13, 200/gm; VC - \$36,000/gm) coupled with inefficient synthesis and reduced efficacy has resulted in the general failure of synthetic analogs; as well, potency of synthetic derivatives has not surpassed the activity of already-available natural bisindoles (especially vincristine). FDA registered chemotherapeutic *Catharanthus* bisindoles are the naturally produced VB, VC and the synthetic, navelbine.

U.S. Patent 4,199,504 and U.S. Patent 4,203,898 describe bridged bis vinca dimers (i.e., tetramers), wherein the single synthetic molecule consists of two dimer subunits linked at the C3 carbon. Such molecules, and derivatives therefrom, are all active anti-mitotic agents and anti tumor agents. Several of the C3 bridged dimers (e.g. "vinca tetramers") possessed demonstrated activity against transplanted tumors in mice *in vivo*, at dose levels comparable to those used with vincristine and vinblastine. All of the Eli Lilly described tetramers are synthetically derived from naturally-occurring vinblastine or vincristine precursors.

A survey of *Catharanthus* alkaloids, both natural and synthetic, that also show demonstrated anti-cancer activity clearly associates the presence of at least one vindoline moiety with the observed activity; thus, vindoline plays a significant role in the expression of anti-cancer activity. Since monomeric vindoline lacks anti-cancer activity, the catharanthine-to-vindoline carbon-carbon bond is integral to expression of activity. Kutney et al. (1976) describes the nature and specificity of the C(18')-C(15) bond (i.e., C18 of the catharanthine monomer to C15 of the vindoline monomer) as regards to natural configuration and activity expression. Dong et al. (1995) further indicate that there is exquisite sensitivity in the structure activity relationships concerning the stereochemistry at C(18'). The inversion of C(18') configuration from *S* to *R* results in a complete loss of activity. Stereochemistry of other carbons in the dimer molecule are similarly critical concerning the interaction with tubulin (Dong et al., 1995.). Finally, the C(18') ester group.

The long history of *Catharanthus* alkaloid investigations has provided an impetus for study of alkaloids produced by other plant genera. Knowledge of metabolic pathways responsible for *Catharanthus* bisindole intermediates has elucidated general terpenoid biosynthetic schemes leading to dimer alkaloids. Within the Apocynaceae, bisindole alkaloids have been reported from

5 *Tabernaemontana* (van der Heijden et al., 1989), *Stemmadenia* (Valencia et al., 1995), and *Strychnos* (Nuzillard et al., 1996). Based on subfamilial relationships in Apocynaceae (Senbald and Bremer, 1996), and close taxa relationships, based on existence of intergenus somatic hybrids (Kostenyuk et al., 1991), there is likelihood of close structural homology of enzymatic pathways leading to bisindole biosynthesis in these taxa. Stevens et al. (1992) investigated shared enzyme

10 characteristics with respect to alkaloid biosynthesis in *Chinchona*, *Tabernaemontana* and *Catharanthus*. While much of the bisindole pathways are shared in common within Apocynaceae, the absence of vindoline as an alkaloid product in *Tabernaemontana* and other allied genera points to the unique biosynthetic attributes of *Catharanthus*. Vinblastine and vincristine along with other dimers that contain a vindoline moiety, are only known to occur in *Catharanthus*. A principal

15 barrier to melding of metabolic pathways, including those in *Catharanthus*, involves barriers to free intergenus genetic exchange. Overcoming these barriers has, in part, provided impetus to plant transformation involving long-distance transferal of *Catharanthus* genes (Kutchen, 1995; Vasquez-Flota et al., 1997).

Aside from the already discussed catharanthine-vindoline dimers, a single additional

20 dimer, composed of two linked vindoline moieties (vindolicine, Figure 3) has been described by Rabaron et al. (1973). Vindolicine ($C_{51}H_{64}N_4O_{12}$, MW=925.086) was isolated from *Catharanthus longifolius*. Rabaron, et al. were the first to recognize the dimeric structure of vindolicine; they also reported the unique UV absorption spectrum of vindolicine. Svoboda et al. (1961) used the name vindolicine to describe a monomeric alkaloid (mol. wt. 457.8) isolated from

25 *Catharanthus roseus*. Based on molecular weight, dimeric vindolicine, as described by Rabaron et al. (1973) has only been isolated from *Catharanthus longifolius*. There are no published reports of anti-cancer activity or known structural derivatives of dimeric vindolicine.

It is desired to identify additional plant alkaloids which will also have biological activity such as anticancer and antifungal activity for mammals and antidisease activity for plants.

SUMMARY OF THE INVENTION

The present invention generally relates to extracts of disease-resistant *Catharanthus* plants, to trimeric and polymeric alkaloids produced by these plants and to the use of the extracts, trimeric alkaloids and polymeric alkaloids as medicinals and anti-biological agents.

- 5 More specifically, the present invention relates to the isolation and identification of novel alkaloid compounds produced by complex *Catharanthus* interspecific hybrids. These hybrids contain in part, germplasm of *Catharanthus roseus*, *C. longifolius*, *C. trichophyllus*, *C. scitulus*, *C. pusilus* and other taxa as described by Veyret (1974), or partial combinations thereof. The nature of some of these hybrids has been previously described in U.S. Patent 5,491,285.
- 10 Biological activity, in the form of *Phytophthora* disease resistance is selected in elite germplasm lines. Disease resistant lines are, in turn, hybridized to enhance biological activity, as detected by increased *Phytophthora* disease resistance. Selected lines, exemplified by enhanced biological activity, are analyzed for alkaloid content using HPLC-MS. Alkaloids are characterized by quantity, quality and correlation with observed disease resistance. Chromatographic peaks are
- 15 identified by physical data such as UV absorption spectra, retention time, mass spectra, fragmentation patterns and NMR profiles. Peak identities are compared with published physical data and comparisons, where possible, with known standards. Through elimination of known alkaloid constituents, novel trimeric and polymeric alkaloids are isolated and disclosed. These trimeric and polymeric alkaloids, as well as the plant extracts, have biological activity, including
- 20 anticancer and antifungal activity.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the basic structure of the bisindole alkaloids, vinblastine (R is methyl) and vincristine (R is formyl).

- 25 Figure 2 shows the structure of the bisindole alkaloid vindesine.

Figure 3 shows the structure of the bisindole alkaloid vindolicine.

Figure 4 represents an HPLC chromatogram of alkaloids in an extract from *Catharanthus roseus* c.v. "Little Pinkie".

Figure 5 represents an HPLC chromatogram of alkaloids in an extract from *Catharanthus*

Figure 6 represents an HPLC chromatogram of alkaloids in an extract from resistant germplasm, line 18652 (in reference to inventor's line number, such as used in U.S. Patent No. 5,491,825).

Figure 7 represents an ultraviolet absorption spectra for compound 1283, vindolicine and vindoline.

Figure 8 represents an HPLC chromatogram of trimer-containing fraction isolated from sample 18652. Numbers indicate molecular weight of selected peaks.

Figure 9 represents an MSⁿ fragmentation of compound 1283 indicating principal fragments formed.

Figure 10 represents a first proposed structure for compound 1283.

Figure 11 represents a second proposed structure for compound 1283.

Figure 12 represents a high-resolution HPLC chromatogram of a trimer fraction in the vicinity of retention times 40-46 minutes.

Figure 13 represents UV spectra of selected peaks indicated in Figure 12.

Figure 14 represents an MSⁿ fragmentation of compound 1351 indicating principal fragments formed.

Figure 15 represents a ¹H-NMR spectrum of compound 1351.

Figure 16 represents a COSY plot of compound 1351.

Figure 17 represents an HSQC plot of compound 1351.

Figure 18A, B and C represent a ¹³C NMR spectrum of compound 1351.

Figure 19 represents a first proposed structure for compound 1351.

Figure 20 represents a second proposed structure for compound 1351.

Figure 21 represents a third proposed structure for compound 1351.

Figure 22 represents a fourth proposed structure for compound 1351.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to extracts of disease-resistant *Catharanthus* plants, to trimeric and polymeric alkaloids produced by these plants and to the use of the extracts, trimeric alkaloids and polymeric alkaloids as medicinals and anti-biological agents.

The present invention also relates to novel alkaloid compounds produced by *Catharanthus* interspecific hybrids. The novel alkaloid compounds are trimeric or polymeric alkaloids, having

three or more monomeric indole alkaloid moieties. One (or two) of the indole alkaloid moieties is a vindoline and/or catharanthine monomer. On the basis of the data presented herein, it is believed that an additional monomer(s) in the novel alkaloid compounds of the present invention is a vindoline-based moiety. In one preferred embodiment, the novel alkaloid compound is a trimer containing three vindoline or vindoline-based monomers. The novel alkaloids of the present invention have molecular weights in the range of about 980 to about 2000 daltons. The alkaloids as isolated from the plant tissues may be saturated or unsaturated with respect to the C6-C7 bond in the vindoline monomer(s). It is preferred that the alkaloids be unsaturated, since it is the unsaturated form which has the highest biological activity. The novel alkaloids of the present invention are isolated from extracts of disease-resistant *Catharanthus* plants.

DEFINITIONS

The present invention employs the following definitions:

“**Alkaloid**” refers to a cyclic organic compound containing nitrogen in a negative oxidation state and which is defined by Bruneton to be of limited distribution among living organisms. (Bruneton, 1995).

“**Catharanthine**” refers to the chemical as described in Chapman and Hall (1997), including racemic and isomeric mixtures thereof.

“**Indole alkaloid**” refers to an alkaloid compound which arises from strictosidine and which possess an indole ring structure. (Bruneton, 1995).

“**Monomer**” or “**subunit**” refer to an alkaloid having a molecular weight of less than 458 daltons, and includes those structures described in Lounasmaa and Galambous (1989).

“**Trimer**” refers to a single chemical entity composed of three indole monomers with a molecular weight of greater than 980 daltons.

“**Vindoline**” refers to the chemical as described in Chapman and Hall (1997), including racemic and isomeric mixtures thereof.

“**Vindoline-based**” refers to a compound which is wholly or in part derived from vindoline or its immediate precursors.

Chromatographic peaks are identified by physical data such as UV absorption spectra; retention

time, mass spectra, fragmentation patterns and NMR profiles. Peak identities are compared with published physical data and comparisons, where possible, with known standards. Through elimination of known alkaloid constituents, novel trimeric and polymeric alkaloids are isolated by HPLC fractionation.

5 More specifically, greenhouse grown and field grown *Catharanthus* plants, selected for enhanced biological activity against *Phytophthora* disease resistance, are assayed for alkaloid content using common methods known to those skilled in the art and as described in the examples below. Any suitable extraction method can be employed to prepare the *Catharanthus* extract. Suitable isolation and separation methods for indole alkaloids have been reviewed by Svoboda
10 (1964), Verpoorte (1987) and U.S. Patent 4,172,077. According to one embodiment of the present invention, mature leaves of the selected *Catharanthus* plants are collected and frozen. The frozen leaves are fractured and ground to a coarse powder and extracted in methanol. The methanol is evaporated and the gum is acid extracted. Any suitable acid can be used for the acid extraction. However, it is preferred to use weak organic acids, such as tartaric or citric acid, to maximize the
15 isolation of unsaturated alkaloids. The acid extracts are basified and the aqueous phase is extracted with an organic solvent. The organic solvent is evaporated to produce the alkaloid sample. The alkaloid sample is redissolved in methanol and subjected to HPLC to further fractionate the extracts. Alternatively, crude alkaloid extracts are subjected to size exclusion ambient pressure column chromatography and HPLC to isolate individual alkaloid compounds. The alkaloid
20 compounds are identified on the basis of their molecular weight, including alkaloids having molecular weights in the range from about 980 to about 2000 daltons. Other physical characteristics are shown for several of the alkaloids of the present invention.

 Extracted samples are analyzed by high pressure liquid chromatography (HPLC), and photo diode array detection (PDA). Analysis methods are discussed by McCloud et al. (1997).
25 Where appropriate, mass spectrometry is used to further clarify molecular structural aspects of detected alkaloids, as used by Verpoorte and Niessen (1994) and Chu et al. (1997). Nuclear magnetic resonance (NMR) is also employed to characterize structures of indole alkaloids using methods known to those in the art and exemplified by Mukherjee et al. (1997) and Andre-Touche et al. (1997). On the basis of the evidence gathered at this time, several possible structures exist

The novel trimeric and polymeric indole alkaloids of the present invention are produced in plants. These naturally-occurring products reflect metabolic activity in stereospecific pathways. HPLC-MS of trimer containing fractions revealed multiple discreet chromatographic peaks for several alkaloids, such as those having m/z 1231.3 and 1241.5. The presence of distinct
5 chromatographic peaks with different retention times yet also with identical mass and UV absorption spectra indicates presence of stereospecific isomeric forms differing in configurational and conformational arrangement. Exquisite sensitivity in structure-activity relationships described for known bisindoles (including VB, VC) suggests that stereospecificity in trisindoles may also be significant in determining bioactivity. Detection of trimeric stereoisomers suggests that
10 presence of multiple forms likely influences observed bioactivity of the trimers.

Yield of trimers is influenced by both genetic and environmental factors. Figure 6 illustrates attainable yield of compound 1283, relative to VB and other associated monomers and dimers. Considering that VB is the starting material from which various medicinal alkaloids are synthesized, the yield of compound 1283 (compare Figures 4 and 6) significantly exceeds that of
15 VB in potential commercial production germplasm approximated by Figure 4.

Attempts to increase alkaloid yield in *Catharanthus roseus* via biotic or physical elicitation have been reported for suspension cell cultures (Godoy-Hernandez and Loyola-Vargas, 1996; Moreno et al., 1996). While increased yield of some monomers (ajmalicine, serpentine) has been reported (Shanks et al., 1998), increased yield of dimers remains unachieved especially in whole
20 plants, despite thirty or more years of intense effort.

Yield of trimers comprising the instant invention of this patent is elicitable. Through manipulation of appropriate biological and physical factors, trimer yields can be increased from ambient levels where the amount of compound 1283 approximates that of VB, to that shown in Figure 6 where the amount of compound 1283 is 13 times greater than VB. Thus, unlike dimers,
25 the production of trimers/polymers can be elicited. Factors responsible for elicitation of trimers include manipulation of the whole-plant pH environment and provision with sulfate. Though not in *Catharanthus*, Sikuli and Demeyer (1997), reported increased hyoscyamine yield in *Datura stramonium* in culture medium in which SO_4^{2-} and K^+ were dominant. Elicitors such as acetylsalicylic acid and salicylic acid do not appear to influence trimer yield.

As shown in Figure 9, compound 1283 (Figure 12), compound 1351 (Figure 13), and other trimers yielded fragments structurally similar

to known dimeric and monomeric indole alkaloids. Based on molecular weight, fractionation of 1283, 1351, VB and VC all yield fragments in common indicating, as expected, close structural homology. Since trimers contain a second vindoline moiety when compared to VB or VC, controlled *in vitro* degradation resulting in the loss of a single vindoline moiety, readily yields VB, VC, or related bisindoles. In addition to synthesis of VB or VC from trimers, the trimers or trimer derivatives, themselves, are used as stereospecific starting materials in the synthesis of novel bisindoles with bioactivity paralleling the activity of known VB and VC derivatives. In addition, the trimeric and polymeric alkaloids of the present invention are used as starting materials for the preparation of bioactive derivatives as described, for example, by Barnett (1978), Conrad et al. (1979), European Patent No. 0,010,458 and U.S. Patent Nos. 5,620,985, 5,024,835, 5,030,620 and 3,352,868.

Disease-resistant plants of U.S. Patent 5,491,285 uniquely produce trimeric and polymeric indole alkaloids. These same plants exhibit profound antibiological activity in the form of elevated disease and pest resistance. Production of dimeric alkaloids, for example VB and VC (see Figure 4), affords mild disease resistance in comparison to vindoline-lacking mutants as shown herein. Thus, the functional role of dimer/trimer/polymer alkaloids *in planta* can be attributed to antibiological defensive action. When extracted, purified and administered to humans, this same antibiological defensive activity of dimers constitutes the long-established role of medicinal *Catharanthus* bisindoles, especially VB and VC. The striking structural similarity of trimers and polymers to bisindoles, coupled with the observed profoundly heightened antibiological expression characterized by trimer/polymer containing plants clearly reflects the potency of trisindole bioactivity. Demonstrated antifungal activity and suppression of predation by diverse pests including mites, insects, and molluscs indicate use of trisindoles and polyindoles in both animals and plants. The usefulness of the trimeric and polymeric alkaloids as antifungal and anticancer agents is demonstrated by antifungal screening assays and anticancer screening assays such as described herein.

The present invention encompasses the use of the novel trimeric and polymeric alkaloids in pharmaceutical and therapeutic modalities for anticancer or antifungal activities. The alkaloids of the present invention can be formulated in pharmaceutical compositions, which are prepared

pharmaceutically acceptable salts of the active agent. These compositions may comprise, in

addition to one of the active substances, a pharmaceutically acceptable excipient, carrier, buffer, stabilizer or other materials well known in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., intravenous, oral, 5 intrathecal, epineural or parenteral, and depending on the therapeutic modality. The compounds are administered in similar manner as other biologically active plant alkaloids, such as vincristine and vinblastine, or they are administered in a similar manner as other antifungal agents.

For oral administration, the compounds can be formulated into solid or liquid preparations such as capsules, pills, tablets, lozenges, melts, powders, suspensions or emulsions. In preparing 10 the compositions in oral dosage form, any of the usual pharmaceutical media may be employed, such as, for example, water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents, suspending agents, and the like in the case of oral liquid preparations (such as, for example, suspensions, elixirs and solutions); or carriers such as starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like in the case of oral solid preparations (such 15 as, for example, powders, capsules and tablets). Because of their ease in administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. If desired, tablets may be sugar-coated or enteric-coated by standard techniques. The active agent can be encapsulated to make it stable to passage through the gastrointestinal tract while at the same time allowing for passage across the blood 20 brain barrier. See for example, WO 96/11698.

For parenteral administration, the compound may be dissolved in a pharmaceutical carrier and administered as either a solution or a suspension. Illustrative of suitable carriers are water, saline, dextrose solutions, fructose solutions, ethanol, or oils of animal, vegetative or synthetic origin. The carrier may also contain other ingredients, for example, preservatives, suspending 25 agents, solubilizing agents, buffers and the like. When the compounds are being administered intrathecally, they may also be dissolved in cerebrospinal fluid.

For topical administration, the active agent is added to a carrier useful for topical administration. The carrier can vary widely depending on the site for topical administration. Formulations suitable for topical administration to the skin may be presented as ointments, creams,

suitable for vaginal administration may be presented as tampons, creams, gels, pastes, foams or spray formulations containing, in addition to the active agent, suitable carriers.

The active agent of the present invention, when used as an anticancer agent, is administered in the same manner as vinblastine or vincristine. Because of severe toxic reactions, 5 vinblastine and vincristine are administered by an IV drip.

The active agent is preferably administered in a therapeutically effective amount. The actual amount administered, and the rate and time-course of administration, will depend on the nature and severity of the condition being treated. Prescription of treatment, e.g. decisions on dosage, timing, etc., is within the responsibility of general practitioners or specialists, and typically 10 takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of techniques and protocols can be found in *Remington's Pharmaceutical Sciences*.

Alternatively, targeting therapies may be used to deliver the active agent more specifically to certain types of cell, by the use of targeting systems such as antibodies or cell specific ligands. 15 Targeting may be desirable for a variety of reasons, e.g. if the agent is unacceptably toxic, or if it would otherwise require too high a dosage, or if it would not otherwise be able to enter the target cells.

Catharanthus seeds resistant to *Phytophthora* have been placed on deposit with the American Type Culture Collection, Manassas, Virginia, under Deposit Accession Number 75636 20 on 14 January 1994 in connection with U.S. Patent No. 5,491,285. All restrictions on the availability of the seed have been lifted in connection with said patent.

EXAMPLES

The present invention is described by reference to the following Examples, which are 25 offered by way of illustration and is not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below were utilized.

EXAMPLE 1

Extraction of *Catharanthus roseus* alkaloids

Seeds of *Catharanthus roseus* were collected and frozen. 100 30 frozen leaves were fractured and ground to a coarse powder and forced-air dried at 10° C for ca.

24 hrs. The dried leaves were extracted in methanol (ca. 1 hr.) and filtered. The filtrate was evaporated to yield a dark gum. The gum was twice extracted for ca. 1 hour with 1% H₂SO₄, the extracts combined, filtered and adjusted to pH 9 by addition of ammonium hydroxide or other suitable base. The aqueous phase was extracted three times with ethyl acetate, methylene chloride or other appropriate organic solvent (see Verpoorte, 1987). The organic extracts were combined, filtered, dried where appropriate over anhydrous sodium sulfate or other suitable agent, and evaporated, yielding the alkaloid sample.

The alkaloid sample was redissolved in a small amount of methanol and analyzed with HPLC and PDA detection utilizing standard methods known to those in the art (see Shanks et al., 1998). Analysis of extracts (Figure 4) revealed the presence of several expected monomeric alkaloids including but not limited to strictosidine, catharanthine and vindoline. Dimeric indole alkaloids were also detected including, but not limited to vinblastine, vincristine, leurosine, leurosidine, 3', 4'- anhydrovinblastine and catharine. PDA detection allowed for quantification and peak purity checking. Peak identities were confirmed by retention times and ultraviolet absorption spectra. Peak identities were validated by comparison with standards of strictosidine, catharanthine, vindoline, vinleurosine, lochnerine, vindolicine, catharine, vinblastine, and vincristine, as well as physical data from the literature, including Sangster and Stuart (1965) and Neuss (1963). Peak areas in Figure 4 indicate relative abundance and composition of selected chromatographic peaks in *Catharanthus roseus* cv. "Little Pinkie."

EXAMPLE 2

Extraction of *Catharanthus longifolius*

Leaves of *Catharanthus longifolius* (Pichon) were prepared and extracted as in the previous example. HPLC analysis revealed the presence of several monomeric and dimeric alkaloids in common with *Catharanthus roseus* (Figure 5). The alkaloid chemistry of minor *Catharanthus* spp. is also reported by Tin-Wa and Farnsworth (1975). In addition to the dimeric alkaloids found in *Catharanthus roseus*, another bisindole, vindolicine (Figure 3) was detected in *C. longifolius*. Rabaron et al. (1973) also found vindolicine in *C. longifolius* and further structurally characterized the dimer molecule as being composed of two vindoline subunits linked

confirmed in the present extracts. To date dimeric vindolicine has only been reported in *C.*

longifolius. Svoboda et al. (1961) identified a monomeric alkaloid having a molecular weight of 457.8 as vindolicine which should not be confused with the dimeric compound of Rabaron et al. (1973), and whose structure is confirmed in Chapman and Hall (1997).

EXAMPLE 3

Interspecific Crosses In *Catharanthus*
as Source of Germplasm for Alkaloid Isolation

Germplasm of *Catharanthus* species was grown to flowering maturity in a greenhouse using methods known to those skilled in the trade. Species included in a complex crossing program are listed in Table 1. Confirmation of hybridity was established by tracking morphological, reproductive and phytochemical traits, including *Phytophthora* disease resistance (see U.S. Patent No. 5,491,285). More than 9,000 hybrid lines, and selections therefrom provided genetic backgrounds which could be assessed for alkaloid content and inheritance patterns. Extreme variance in morphological and reproductive forms suggested that a parallel variability in phytochemical biotypes might also be present.

TABLE 1

Species and Interspecies Hybrids Investigated for Alkaloid Content.

15	<i>Catharanthus roseus</i>
	<i>C. longifolius</i>
	<i>C. trichophyllus</i>
	<i>C. scitulus</i>
	<i>C. pusilus</i>
20	<i>C. roseus</i> biotypes ¹
	<i>C. roseus</i> cultivars ²
	<i>C. roseus</i> x <i>longifolius</i> ³
	<i>C. roseus</i> x <i>trichophyllus</i> ³
	<i>C. roseus</i> x <i>scitulus</i> ³
25	<i>C. roseus</i> x <i>roseus</i> biotypes ³
	<i>C. roseus</i> x <i>longifolius</i> x <i>trichophyllus</i> x <i>scitulus</i> x <i>roseus</i> biotypes x <i>roseus</i> cultivars ^{3,4}

¹ Non-typical forms of *C. roseus* see U.S. Patent No. 5,491,285. Includes *Phytophthora* resistance gene of U.S. Patent No. 5,491,285.

² Including but not limited to cultivars in U.S. Patent No. 5,491,285, Table 1.

³ Includes cross and reciprocal, backcrosses, and multiple succeeding generations including backcrosses.

⁴ Includes in excess of 12 generations and more than 9,000 breeding crosses.

EXAMPLE 4

Indicated in Table 1, germplasm of the *Phytophthora* resistance gene of U.S. Patent No. 5,491,285, was included in the crossing program. Extraction of alkaloids from this

resistance stock utilized methods of Example 1. A chromatogram of alkaloids derived from a line containing the resistance gene is shown in Figure 6. PDA evaluation of chromatographic peaks revealed the monomeric and dimeric alkaloids characteristic of *Catharanthus roseus*. As well, resistance germplasm also produces small amounts of vindolicine, as indicated by the peak shown in Figure 6. Detailed examination of other chromatographic peaks indicated their presence in the resistance line, *C. roseus*, *C. longifolius*, or combinations thereof. A chromatographic peak at an approximate retention time of 43.3 minutes (Figure 6) was characteristic of lines containing the resistance gene; this same peak was not present in *C. roseus* of Example 1 or *C. longifolius* of Example 2. Exhaustive attempts to detect this peak using concentrations from large amounts of plant material and other methods known to those skilled in the art of HPLC, failed to reveal the presence of the peak with retention time 43.3 min. in any examined germplasm of *C. roseus* or *C. longifolius*. Examination of numerous lines containing the resistance gene of U.S. Patent 5,491,285 revealed a perfect correlation with presence of the resistance gene; conversely, lines segregating for sensitivity (non-resistant) lacked detectable signal for the chromatographic peak at approximately 43.3 minutes.

Absolute amounts of the novel peak, as indicated by relative comparisons of peak areas of known dimers, especially vinblastine, indicated total amounts of the novel compound were variable, dependent on the resistant line examined.

EXAMPLE 5

Extraction of Commercial *Catharanthus* Lines

Commercially available *Catharanthus* germplasm, including but not limited to those cultivars listed in Table 1 of U.S. Patent No. 5,491,285 were examined for presence of the novel peak described in Example 4. All obtainable cultivar germplasm failed to contain the novel compound of Example 4, using the highest attainable resolution capable with PDA instrumentation (Model 996, Waters Corporation, Milford, MA). All available *Catharanthus* germplasm lines available from the United States Dept. Agriculture (GRIN, Beltsville, MD) similarly failed to possess the novel compound. To date, the novel compound as shown by the chromatographic peak at an approximate retention time of 43.3 minutes (Figure 6) and as further characterized herein has

EXAMPLE 6

Peak Purity

As indicated, all resistant lines expressing *Phytophthora* disease resistance, characterized by the methods expounded in U.S. Patent No. 5,491,285, possess finite quantities of the novel alkaloid described in Example 4. In order to better exemplify the physical characteristics of this novel compound, selected genetic lines such as line 18652 were grown in the greenhouse and field to produce larger amounts of the compound in question. Larger samples were HPLC injected and the eluate corresponding to the 43.3 minute peak was collected, using common HPLC methodology known to those in the art. The peak was re-extracted at low pH, a portion again subjected to HPLC analysis and analyzed for peak purity utilizing common peak analysis protocols (Millennium ver. 2.15, Waters, Milford, MA). Results indicated a single compound with a ultraviolet absorption as indicated in Figure 7, which compares the UV absorption spectra of compound 1283 with vindolicine and vindoline. HPLC analysis of additional portions at high pH (7.5) further confirmed the presence of a single pure compound, characterized by a slight bathochromatic shift, a feature common to indole alkaloids (Sangster and Stewart, 1965). Comparison of the UV spectrum (Figure 7) of the novel compound with existing spectra libraries (Sangster and Stewart, 1965; Neuss, 1963) failed to reveal a match, further indicating novelty; the spectrum is, however, reasonably close to that of vindoline (Neuss, 1963) and vindolicine (Rabaron et al., 1973) suggesting close structural affinity.

20

EXAMPLE 7

Mass Spectroscopy: Molecular Weight

Portions of the pure compound obtained in Example 6 were analyzed with mass spectroscopy (Finnigan, San Jose, CA). Results confirmed a single-charged pure compound of m/z 1283.5 for the novel compound. Analyses of multiple samples derived from different germplasm lines resistant to *Phytophthora* including but not limited to 18652 and 18795 and isolated with variant extraction methodologies, such as disclosed in Svoboda (1964), Verpoorte (1987) and U.S. Patent No. 4,172,077, repeatedly yielded the same compound. It was further found that any method suitable for extracting indole alkaloids from plant tissue repeatedly yielded

25

Table 2 indicates known molecular weights for alkaloids isolated from *Catharanthus*, allied Apocynaceae genera and other organisms. Notably, the highest molecular weight for any known alkaloid isolated from any *Catharanthus* species is 925 (vindolicine, Chapman and Hall, 1997). Vindolicine also possesses the largest molecular weight reported for any indole alkaloid.

5 Stepwise increase in molecular weights (Table 2) suggested the novel compound might be a polymeric alkaloid composed of multiple monomeric units. Since all known *Catharanthus* dimers are composed of catharanthine and/or vindoline moieties, possible molecular weights were calculated for trimers based on combinations of catharanthine and vindoline moieties derived from both vinblastine and vincristine (Table 3). The extreme similarity of the observed m/z (1283.5)
10 and the calculated molecular weight of trimers containing a single catharanthine and two vindoline moieties strongly implicated the novel compound as a trimer indole alkaloid.

TABLE 2

15 Molecular Weights¹ of Naturally-Occurring Alkaloids

	<u>Alkaloid</u>	<u>Source</u>	<u>Family</u>	<u>Class</u> ²	<u>Molecular Wt.</u>
	vindoline	<i>Catharanthus</i>	Apocynaceae	M	456
	catharanthine	<i>Catharanthus</i>	Apocynaceae	M	336
	vincristine	<i>Catharanthus</i>	Apocynaceae	D	825
20	vinblastine	<i>Catharanthus</i>	Apocynaceae	D	811
	leurosine	<i>Catharanthus</i>	Apocynaceae	D	809
	catharine	<i>Catharanthus</i>	Apocynaceae	D	823
	vinamidine	<i>Catharanthus</i>	Apocynaceae	D	825
	3, 4 -anhydro-	<i>Catharanthus</i>	Apocynaceae	D	793
25	vinblastine				
	vindolicine	<i>Catharanthus</i>	Apocynaceae	D	924 ³
	conoduramine	<i>Tabernaemontana</i>	Apocynaceae	D	703
	voacamine	<i>Tabernaemontana</i>	Apocynaceae	D	703
	tabernaemegantinine	<i>Tabernaemontana</i>	Apocynaceae	D	762 ⁴
30	michellamine B	<i>Ancistrocladus</i>	Ancistrocladaceae	D	770
	Hamacanthin A	<i>Hamacantha</i>	(marine sponge)	D	486
	Panganensine	<i>Strychnos</i>	Loganiaceae	D	587

¹ Reported as molecular weight of free base.

35 ² Monomer (M) or dimer (D).

³ Highest molecular weight reported for any naturally-occurring indole alkaloid

TABLE 3

Theoretical Molecular Weight of Trimers¹.

	<u>Trimer Combination</u>	<u>Expected Molecular Weight</u>
5	VB+ V(VB)	1265.5
	VB+ V(VC)	1279.5
	VC+ V(VB)	1279.5
	VD+ C(VB)	1279.5
	VC+ V(VC)	1293.5
10	VD+ V(VB)	1379.6
	VD+ V(VC)	1393.6
	V(VC)+ V(VC)+V(VB)	1393.5
	V(VC)+ V(VC)+V(VC)	1407.5
	VB+ VC	1633.9
15	VB+ VD	1734.0
	VC+ VD	1748.0

¹ abbreviations: VB =vinblastine, VC = vincristine, VD= vindolicine, V(VB) = vindoline moiety as in vinblastine or vindolicine, V(VC) = vindoline moiety as in vincristine, C(VB) = catharanthine moiety as in VB or VC.

EXAMPLE 8

Liquid Chromatography: Trimer Fractions

Endo et al. (1987) used Sephadex LH-20 to separate *Catharanthus roseus* dimeric alkaloids from monomers. Crude whole alkaloid extracts obtained as described in Example 4 were subjected to size exclusion ambient pressure column chromatography using Sephadex LH-20. As is known to those in the art, separations are accomplished based on molecular size, which in turn, is dependent on molecular weight. Sequential column chromatography fractions were collected and analyzed by HPLC as described in Example 4. As expected, the earliest size-exclusion fractions were devoid of all dimeric alkaloids (vinblastine, etc.) but did contain the heavier novel trimeric alkaloid (m/z 1283) at its expected retention time. Moreover, additional peaks were detected in the 1283-containing fraction, indicating presence of previously undetected trimers or polymers. In non-fractional analyses (as in Figure 6) these additional trimer peaks had been hidden by co-eluting monomers of substantially greater abundance. Combined UV spectral data and retention times confirmed the identity of compound 1283 and differentiated the other trimer/polymer compounds. Increased amounts of compound 1283 were detected in the fractions.

EXAMPLE 9

HPLC-MS

Trimer-rich fractions obtained as described in Example 8 were subjected to further analysis by combined HPLC-mass spectroscopy (HPLC-MS). Peaks eluting from the HPLC column are directly shunted to mass detection, thereby correlating retention time, peak purity, and UV absorption spectra with molecular weight and fractionation patterns. Figure 8 shows the HPLC chromatogram of a trimer fraction with corresponding selected molecular weights (m/z) as indicated. Significant peaks having molecular weights of 982, 1163, 1231, 1241, 1281, 1283 and 1351 are shown in this Figure. A complete analysis of the chromatogram showed that discrete alkaloids with m/z 982, 1127, 1145, 1154, 1163, 1182, 1193, 1231, 1241, 1247, 1253, 1263, 1269, 1279, 1281, 1283, 1299, 1305, 1325, 1351, 1352, 1422, 1453, 1456, 1533, 1535, 1653, 1738, 1747, 1766, 1870, 1958, and 1973 were also detected. These alkaloids were further characterized with additional physical data, including mass fragmentation patterns, confirming their identity as indole alkaloids related to VB and VC. All of these peaks exceed the highest known molecular weight for any reported indole alkaloid (vindolicine, Table 2, mol. wt. 924). Therefore, the compounds of these peaks comprise a novel class of trimer/polymer alkaloids constructed, at least in part, from monomeric catharanthine and vindoline entities. As is known to those in the art, baseline perturbations likely reflect additional trimer/polymer alkaloids whose HPLC-MS signals were below resolution levels of the instrumentation employed. Thus, additional trimer peaks were detected and quantified although they were not fully characterized.

EXAMPLE 10

Analysis of Extracts for Artifacts or Degradation

As with other organic constituents, alkaloids are subject to degradation and autolysis, dependent on extraction, storage and analysis methods employed. Verpoorte (1987) has suggested that halogenated solvents can induce artifacts in indole alkaloids. Trimer containing fresh leaves were subjected to repeated extractions using diverse solvents, acids, bases, solid phase extraction and other methods known to those in the art, including those described in Svoboda (1964), Verpoorte (1987) and U.S. Patent No. 4,172,077. Results from these, as well as other known

natural products rather than artifacts induced by specific analysis protocols.

Sethi and Thimmaiah (1985) and Thimmaiah and Sethi (1985) reported on degradation of vinblastine and vincristine, induced by both biotic and abiotic factors. In all instances, degradation products were characterized by lower molecular weights than the parent compounds.

Catharanthus bisindoles are also light and heat labile (U.S. Patent No. 4,831,133; Bommer et al., 1964). Trimer containing leaf samples were intentionally subjected to adverse drying conditions, extracted and subsequently analyzed for dimer and trimer alkaloid content. Loss of trimer peaks was concomitant with loss of dimer peaks, further substantiating that trimer compounds are natural products which are chemically reactive in manners similar to other indole alkaloids. Induced degradation of bisindole standards and trimer extracts similarly results in loss of higher molecular weight components, further substantiating that trisindoles are naturally synthesized *in planta* products.

EXAMPLE 11

C6-C7 Saturation of Vindoline Moieties

Catharanthus bisindoles, including vinblastine, vincristine, vindesine and derivatives therefrom, are known to undergo reduction of the 6,7 double bond in the vindoline moiety, resulting in a +2 change in molecular weight (see Figure 1). Noble et al. (1967) described the resulting increase of molecular weight (+2) in dihydrovinblastine compared to the unsaturated vinblastine. Dihydrovinblastine can be produced by hydrogenation of vinblastine under acetic conditions (U.S. Patent No. 3,352,868). Bieman (1964) demonstrated a +2 increase in molecular weight for other dihydro-bisindole alkaloids containing a vindoline moiety. Reduction of the 6,7 double bond in vinblastine (U.S. Patent No. 3,352,868) results in a substantial (13x) loss of anti-cancer activity. Reduction of the 6,7 double bond in vindesine similarly results in substantial reduced anti-cancer activity (Barnett et al., 1978).

High resolution mass spectroscopy of the 1283 compound revealed a m/z of 1283.5850. As indicated in Table 3, trimers composed of a single catharanthine and two unsaturated vindoline moieties would be expected to have a molecular weight of 1279.5. Saturation of the 6,7 double bond of both vindoline moieties would yield the observed molecular weight of 1283.5. As explained in Example 1, trimer containing leaves were extracted in a strong mineral acid (H₂SO₄).

High resolution mass spectroscopy of the 1283 compound revealed the presence of two small chromatographic peaks immediately preceding elution of the 1283 peak.

The previously unanalyzed peaks had m/z of 1279.5 and 1281.5, respectively. When additional trimer containing leaf samples were extracted with tartaric acid, a weak organic acid (Svoboda, 1964), instead of sulfuric acid, the abundances of the 1279.5 and 1281.5 peaks increased relative to the area of the 1283.5 peak. Thus, naturally occurring trisindoles with m/z 1279.5, 1281.5 and 1283.5 were extracted and isolated when tartaric acid was used in place of sulfuric acid. Similar results are obtained when citric acid, another weak organic acid, is used in place of sulfuric acid in the extraction of trimer containing leaves. The respective relative yields were dependent upon extraction parameters and methodologies employed. Detailed analysis of other trimers, as isolated in Example 9, indicated the presence of unsaturated and saturated forms, dependent on the vindoline moieties in the compound. Similarly with the results achieved using tartaric acid or citric acid for extraction with respect to compound 1283, unsaturated trimers are isolated with respect to the saturated trimers identified in Example 9.

Barnett et al. (1978) reported the formation of vindesine N_b -oxides upon prolonged storage of vindesine free base. The N_b -oxide, characterized by a net increase of $m/z +16$ (corresponding to an added oxygen) could be reduced back to the free base form. HPLC-MS analysis of a trimer enriched fraction showed that the primary constituent comprised compound 1283 and that two additional peaks, both with m/z 1299 eluted earlier. These earlier eluting peaks are consistent with a lower pK_a expected of the 1283 N_b -oxides. That two distinct peaks with $+16 m/z$ were found reflects isomeric forms of the N_b -oxides, chromatographically discernable because of slight differences in pK_a . Existence of 1283 N_b -oxides (m/z 1299) further verifies the existence of vindoline moieties in the trimer compounds.

EXAMPLE 12

Characterization of Compound 1283

A detailed comparison of UV absorption spectra for compound 1283, vindoline, and vindolicine is provided in Figure 7; all samples were analyzed under identical solvent and pH conditions. Spectral analysis reveals close similarity between the three compounds. Mass spectral analysis (MS^n , Finnigan, San Jose, CA) of an infused sample of compound 1283 revealed the fragmentation indicated in Figure 9. Fragmentation patterns for other trimers and standards of VB

Figure 10. As known to those in the art of chemical structure determinations, other structural

arrangements may also be present other than that indicated in Figure 10, including the proposed structure shown in Figure 11, that can explain the observed physical characteristics and bioactivity of compound 1283.

High resolution HPLC of a trimer containing fraction extracted from line number 18733 is shown in Figure 12. Selected peaks in near vicinity to compound 1283 (retention time 42.386 in this trace) are integrated as shown. Corresponding UV absorption spectra for integrated peaks of Figure 12 are shown in Figure 13 (marked by retention time). Discreet chromatographic peaks with such close retention times and similar absorption spectra suggest multiple detected isomeric or racemic forms. For example, peaks with retention times 42.38 (compound 1283) and 43.55 possess essentially identical UV spectra. These molecular forms likely vary structurally by bond angle or substitution position but may not possess differing molecular weights. Detection of distinct chromatographic peaks demonstrates that trimer metabolic pathways generate numerous molecular forms in addition to those indicated in Figures 10-11. These closely related structural forms vary by relative abundance as indicated in Figure 12. Collectively, Figures 10-13 indicate presence of multiple molecular forms generally represented in Figures 10-11, though not having exactly the same trimeric structural configuration as illustrated.

EXAMPLE 13

Characterization of Compound 1351

Using methods as described in Example 12, compound 1351 was further characterized.. The ultraviolet absorption spectrum is characterized as Λ_{\max} 215.9, 245.2, 314.9. Principal MS fragmentation products of compound 1351 are shown in Figure 14.

A composite sample of compound 1351 was obtained by combining multiple HPLC fractions containing the 1351 peak. An approximate 3 mg sample of compound 1351 was analyzed by NMR to yield ^1H NMR, COSY, HSQC, ^{13}C NMR, and MS data as described below.

^1H NMR (Figure 15):

Key pieces of structural information derived from the ^1H NMR spectrum are listed below:

1. There are five equally integrated aromatic protons (~6.2-7.6 ppm). Because all are indicated as singlets, the trimer may not contain a catharanthine moiety, and instead

triplets). This supports the trimer structure based on three vindoline based subunits.

3. There are five methyl groups attached to oxygen as either methoxy moieties or esters (~3.6 ppm singlets).
4. There are three methyl groups attached directly to a carbonyl carbon (~2.8 ppm singlets).
5. There are six olefinic protons:
 - 3 as a ~5.9 ppm multiplet, corresponding to the vindoline carbon position 7 in all three subunits (see Figure 1 for numbering convention in the vindoline moiety).
 - 3 as doublets from 5.5-5.7 ppm, corresponding to position 6 in all three subunits.
6. There are three methyl groups attached to nitrogen (~1.99-2.05 ppm singlets).

COSY (Figure 16):

The COSY data plots ^1H NMR spectra on both X and Y axes. Correlations between protons indicate that they are attached to adjacent carbons. COSY information is summarized below:

1. The aromatic signals showed no correlations. This was expected because they were singlets in the ^1H NMR spectrum and so should have no protons attached to adjacent carbons.
2. The molecule is complex in the aliphatic region.
3. One of the methyl groups of the ethyl side chains is in a different chemical environment from the other two. The protons at 0.6 ppm show correlations to protons resonating at ~1 ppm and at 1.75 ppm. The protons at 0.8 ppm show correlations to protons resonating at ~1 ppm and 1.90 ppm. However, the protons at 0.7 ppm show correlations to protons resonating at 1.6 and 1.85 ppm. This indicates that there is something different near the ethyl side chain between this monomer and the other two. While this difference could be due to conformational variance, it could also indicate some different functionality in the vicinity of this side chain.

HSQC (Figure 17):

HSQC is a two dimensional experiment that correlates the peaks of a ^1H NMR spectrum

1. 35 proton bearing carbons are detected (non-protonated carbons do not appear in HSQC).
2. The C 17 position has a unique chemical shift (~95 ppm) in the ^{13}C spectrum. The HSQC shows only two correlations from aromatic protons (6.2 and 6.3 ppm) to carbons resonating at ~95 ppm, not three. Since the molecule is a trimer, this implies that one of the monomer units is not protonated at the C 17 position, while the other two are. It is also possible that an alcohol is attached at this C 17 position and the connection to the other monomer units is at the C 3 position, as in other parts of the trimer molecule.
3. Observed correlations also support the presence of the ethyl side chains in the molecule.

^{13}C NMR (Figure 18):

Despite the undesired signal to noise ratio of Figure 18, the NMR software was able to evaluate 55 of the 74 detected signals. Table 4 summarizes salient aspects derived from the ^{13}C NMR.

TABLE 4
Summarized ^{13}C NMR Information

number of signals expected	type of carbon responsible for this signal	expected chemical shift	observed
6	carbonyl carbons	~170-172 ppm	5 signals in this region
4	Ar-O-R (aromatic carbons with oxygens attached to them, ie. Ar-OMe or Ar-OH)	~150-160 ppm	5 signals in this region
3	position 18 carbons	~150 ppm	
3	methylys that are part of the ethyl side-chain	~7-9 ppm	3 signals in this region

MS:

analyzed by HPLC and MS. A degradation product with m/z 457 (vindoline) was readily

detected; thus, a single vindoline unit can be generated from degradation of compound 1351. Another degradation product with m/z 937, corresponding to vindoline+vindoline+acetate, was readily detected. Thus, upon exposure to adverse conditions, the 1351 compound can degrade to release either one separate or two adjacently linked vindoline moieties.

Combined physical data indicate a possible structure for compound 1351 as shown in Figure 19. As known to those in the art of chemical structure determinations, other structural arrangements may also be present beyond that indicated in Figure 19, including the proposed structures shown in Figures 20-22. Just as with compound 1283, high resolution HPLC of trimer enriched fractions revealed multiple though small discrete chromatographic peaks near the primary 1351 peak of Figure 8. Several of these trace peaks possess UV spectra substantially similar to that of compound 1351. Thus, though not of equal relative abundance, multiple isomeric or racemic forms of compound may coexist encompassing at least those structures indicated in Figures 19-22.

EXAMPLE 14

Characterization of Additional Trimers and Polymers

Additional trimers and polymers as indicated, in part in Figure 8 and Example 9, are described in Table 5. Extraction parameters, specific for indole alkaloids, yielded these compounds which were additionally characterized by the indicated physical data. Combined evidence indicates that these compounds are indole alkaloids. Based on similarity to calculated molecular weights for multiple monomeric units (Table 3), the compounds of Table 5 are trimers or polymers composed of multiple monomeric units, especially catharanthine and vindoline.

TABLE 5

Characteristics of Trimers and Polymers

<u>Molecular weight (m/z)</u>	<u>UV absorption (λ_{max})</u>
982 1127	213.6, 260.5, 309.0
1182	213.6, 249.9, 309.0

	1193	214.8, 258.2, 306.6
	1231	214.8, 246.4, 316.1
	1241	213.6, 249.8, 309.0
	1247	215.9, 262.5
5	1253	
	1263	
	1269	214.8, 253.5, 313.7
	1279	
	1281	
10	1283	
	1299	212.4, 251.1, 305.4
	1305	
	1325	215.9, 271.1
	1347	
15	1349	
	1351	
	1352	
	1422	213.6, 255.8, 307.9
	1453	
20	1456	
	1533	
	1535	
	1653	
	1738	
25	1747	
	1766	
	1870	
	1958	
	1973	
30		

EXAMPLE 15

Non-Vindoline Containing Mutants

In the course of investigating alkaloid production in selected genetic lines and correlating observed alkaloid content with disease resistance, a mutation was discovered in a selected line (15453) that completely lacks monomeric vindoline. The trait is governed by a single recessive allele. Lines that express the mutation lack any detectable vindoline signal. Vindoline is a component of all known dimers, and is an expected component of trimers and polymers (as described above). Leaves were subjected to alkaloid extraction as describe above. It was found that *Catharanthus* lines and some *Ipomoea* lines (e.g., 15453) that do not contain vindoline as a monomer, vindoline-lacking plants (thus also lacking dimers and

trimers/polymers) are especially sensitive to disease; they are also prone to infestation by mites, aphids and other common greenhouse pests. Keeping these plants alive in normal greenhouse conditions represents a significant challenge since opportune diseases and pests readily attack and kill the plants.

5

EXAMPLE 16

Assessment of Specific Chemical Bioactivity of Trimeric Alkaloids

Enriched trimer fractions, as described in Example 8 and as extracted using tartaric acid, and collection of specific chromatographic peaks yielding discreet HPLC eluates (specific chemicals) according to the procedure described in Example 9 are analyzed for anti-fungal activity against cultures of *Phytophthora*, generally as described by Kato et al. (1996). The fractions or specific chemicals are applied to inert filter discs followed by evaporation of the solvent. The discs are then applied to axenic fungal or microbial cultures in petri dishes and incubated under conditions suitable for normal growth. The antifungal or antimicrobial activity of the individual fractions or specific compounds is seen by measuring the zones of growth inhibition. It is found that the unsaturated trimeric or polymeric alkaloid compounds, e.g., compound 1279 possess antifungal activity.

EXAMPLE 17

20

Assessment of Specific Chemical Bioactivity of Trimeric Alkaloids

Purified alkaloids isolated from *Catharanthus* tissues are submitted to the National Cancer Institute (NCI) for determination of anti-cancer activity in their *in vitro* Anticancer Drug Discovery Screen (Boyd and Paull, 1995). Purified compound 1283 was tested by NCI, and as expected from its saturation, was not highly active in the screening assay. Purified compounds 1279 and 1281 are tested by NCI. Compound 1279 is found to be active in the screening assay and compound 1281 is found to have intermediate activity to that seen for 1279 and 1283.

It will be appreciated that the methods and compositions of the instant invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent to the artisan that other embodiments exist and do not depart from the spirit of

LIST OF REFERENCES

- Aerts and DeLuca (1992). *Plant Physiol.* 100:1029-1032.
- Andre-Touche et al. (1997). *Agric. Food Chem.* 45:2148-2152.
- Barnet et al. (1978). *J. Med. Chem.* 21:88-96.
- 5 Bieman (1964). *Lloydia* 27:397-405.
- Bommer et al. (1964). *J. Am. Chem. Soc.* 86:1439-1440.
- Boyd and Paull (1995). *Drug Development Res.* 34: 91-109 (1995).
- Bruneton (1995). *Pharmacognosy Phytochemistry Medicinal Plants*, LaVoisier, Paris.
- Chapman and Hall (1997). *Dictionary of Natural Products*, release 6.1.
- 10 Chu et al. (1997). *J. Liq. Chrom & Related Technol.* 20:1159-1174.
- Conrad et al. (1979). *J. Med Chem.* 22:391-400.
- DiCosmo and Misawa (1995). *Biotechnology Advances* 13:425-453.
- Dong et al. (1995). *Phytochemistry* 40:1821-1824
- Dyke and Nelson (1977). *Cancer Treat. Rev.* 4:135.
- 15 Endo et al. (1987). *Planta Medica* 53:479-482.
- Endo et al. (1988). *Phytochemistry* 27:2147-2149.
- Farnsworth (1961). *Lloydia* 24:105-139.
- Godoy-Hernandez and Loyola-Vargas (1997). *Plant Cell Reports* 16:287-290.
- IGT pharma (1998). <http://www.biotech.bcca/bcba/igt/>.
- 20 Kato et al. (1996). *Biosci. Biotech. Biochem.* 60:2081-2083.
- Kostenyuk et al. (1991). *Theor. Appl. Genet.* 82:713-716.
- Kutchan (1995). *The Plant Cell* 7:1059-1070.
- Kutney et al. (1976). *Helvetica Chimica Acta* 59:2858-2882.
- Kutney (1990). *Natural Products Report* 7:85-103.
- 25 Lounasmaa and Galambous (1989). In *Progress in the Chemistry of Organic Natural Products*, Herz et al., eds., 55:89-115.
- McCloud et al. (1997). *Phytochemical Analysis* 8:120-123.
- McMahon (1963). *Experientia*, 19:434-435.
- Meijer et al. (1993). *J. Plant Res.* 3:145-164.
- 30 Moreno et al. (1995). *Plant Cell Tissue and Organ Culture* 42:1-25

- Neuss (1963). *Lilly Collection of Physical Data of Indole and Dihydroindole Alkaloids*, Neuss, ed. Lilly Research Labs, Indianapolis.
- Noble et al. (1967). *Cancer* 20:885-890.
- Nuzillard, et al., *Phytochem.* 43:897-902.
- 5 Rabaron et al. (1973). *Plant. Med. Phytother.* 7:53-58.
- Remington's Pharmaceutical Sciences*, 18th Ed., Mack Publishing Co., Easton, PA (1990).
- Sangster and Stuart (1965). *Chem. Rev.* 65:69-130.
- Sennblad and Bremer (1996). *Pl. Syst. Evol.* 202:153-175.
- Sethi and Thimmaiah (1985). *Cancer Res.* 45:5382-5385.
- 10 Shanks et al. (1998). *Biotechnology and Bioengineering* 58:333-338.
- Sikuli and Demeyer (1997). *Plant Cell Tissue and Organ Culture* 47:261-267.
- Sottomayor et al. (1996). *Plant Cell and Environment* 19:761-767.
- Sottomayor et al. (1997). *Enzyme and Microbial Technology* 21:543-549.
- Stevens et al. (1992). *Plant Physiol. Biochem.* 30: 675-681.
- 15 St-Pierre and DeLuca (1995). *Plant Physiol.* 109:131-139.
- Svoboda et al. (1961). *J. Pharm. Sci.* 50:409-413.
- Svoboda (1964). *Lloydia* 27:299-301.
- Svoboda and Blake (1975). In: *The Catharanthus Alkaloids*, Taylor and Farnsworth, eds., Marcel Dekker, New York, pp. 45-83.
- 20 Taylor and Farnsworth (1975). *The Catharanthus Alkaloids*, Marcel Dekker, New York.
- Thimmaiah and Sethi (1985). *Cancer Res.* 45:5386-5389.
- Tin-Wa and Farnsworth (1975) In: *The Catharanthus Alkaloids*, Taylor and Farnsworth, eds., Marcel Dekker, New York.
- Valencia, et al. (1995). *J. Nat. Prod.* 58:134-137.
- 25 Van Beek and Gessel (1988). In *Alkaloids: Chemical and Biological Perspectives*, Pelletier, ed., Wiley & Sons, New York.
- van der Heijden et al. (1989). *Biotechnology in Agriculture and Forestry* 7:506-523.
- Vasquez-Flota et al. (1997). *Plant Molecular Biology* 34: 935-948 (1997)).
- Verpoorte (1987). In *Indole and biogenetically related alkaloids*, Phillipson and Zenk, eds., Academic Press, New York, pp. 91-112.
- 30 Verpoorte and Niessen (1994). *Phytochemical Analysis* 5:217-232.
- Veyret et al (1974). *Candollea* 29:297-307.

Patents and Published Applications

European Published Patent Application No. 0,010,458

PCT Published Patent Application No. WO 96/11698.

U.S. Patent No. 3,352,868

5 U.S. Patent No. 3,932,417

U.S. Patent No. 4,172,077

U.S. Patent No. 4,199,504

U.S. Patent No. 4,203,898

U.S. Patent No. 4,303,584

10 U.S. Patent No. 4,375,432

U.S. Patent No. 4,479,957

U.S. Patent No. 4,831,133

U.S. Patent No. 5,024,835

U.S. Patent No. 5,030,620

15 U.S. Patent No. 5,047,528

U.S. Patent No. 5,491,285

U.S. Patent No. 5,620,985

WHAT IS CLAIMED IS:

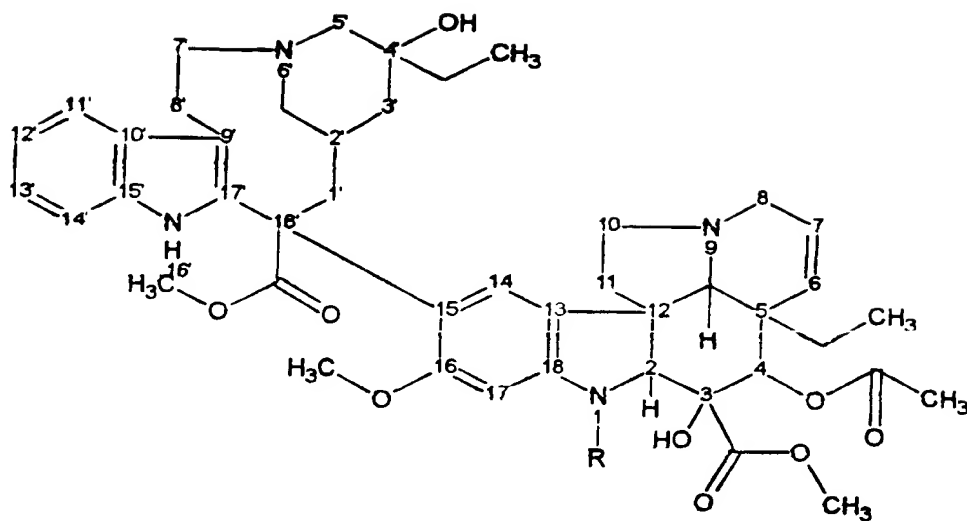
1. An indole alkaloid compound having a molecular weight greater than about 980 daltons.
- 5 2. A trimer alkaloid compound having a molecular weight of greater than about 980 daltons, having at least one vindoline or vindoline-based monomer.
3. A compound isolated from a *Catharanthus* plant having a molecular weight in the range of about 980 to about 2000 daltons, having at least one vindoline or vindoline-based
10 monomer.
4. The compound of claim 2, having a second vindoline or vindoline-based monomer.
5. The compound of claim 4, having a third monomer selected from the group consisting of
15 vindoline, catharanthine, vindoline-based and other monomers.
6. The compound of any one of claims 2 to 4, having a catharanthine monomer.
7. The compound of claim 4, having a molecular weight selected from the group of molecular
20 weights set forth in Table 5.
8. The compound of claim 4, having a molecular weight of about 1279 daltons.
9. The compound of claim 4, having a molecular weight of about 1281 daltons.
25
10. The compound of claim 4, having a molecular weight of about 1283 daltons.
11. The compound of claim 4, having a molecular weight of about 1347 daltons.
13. The compound of claim 4, having a molecular weight of about 1351 daltons.

14. An extract of a *Catharanthus* plant, said extract containing one or more compounds, having a molecular weight in the range of about 980 to about 2000 daltons, having at least one vindoline or vindoline-based monomer.
- 5 15. An extract of a *Catharanthus* plant resistant to *Phytophthora*, said extract containing one or more compounds, having a molecular weight in the range of from about 980 to about 2000 daltons, having at least one vindoline or vindoline-based monomer.
- 10 16. The extract of claim 14, having a second vindoline or vindoline-based monomer.
17. The extract of claim 14, wherein said compound further has a third indole monomer, selected from the group consisting of vindoline, catharanthine, vindoline-based and other monomers.
- 15 18. The extract of claim 14, having a compound with a molecular weight of from about 1279 to about 1283.
19. The extract of claim 14, having a compound with a molecular weight of from about 1347 to about 1351.
- 20 20. A trimer compound isolated from a *Catharanthus* plant resistant to *Phytophthora* having a molecular weight greater than about 980 daltons.
- 25 21. A compound isolated from a *Catharanthus* plant resistant to *Phytophthora* having a molecular weight in the range of from about 980 to about 2000 daltons, having at least one vindoline or vindoline-based monomer.
22. A derivative or a pharmaceutically acceptable salt of the compound of any one of claims

23. A pharmaceutical composition comprising an effective amount of the compound of any one of claims 3, 6, 20 and 21 or a pharmaceutically acceptable salt thereof as an active agent and a pharmaceutically acceptable carrier.
- 5 24. A pharmaceutical composition comprising an effective amount of the extract of any one of claims 14 to 19 as an active agent and a pharmaceutically acceptable carrier.
- 10 25. A pharmaceutical composition comprising an effective amount of the derivative or pharmaceutically acceptable salt of claim 22 as an active agent and a pharmaceutically acceptable carrier.
26. A method comprising administering the pharmaceutical composition of claim 23 to an individual in need of said agent.
- 15 27. A method comprising administering the pharmaceutical composition of claim 24 to an individual in need of said agent.
- 20 28. A method comprising administering the pharmaceutical composition of claim 25 to an individual in need of said agent.
- 25 29. A method comprising administering the pharmaceutical composition of claim 23 to a plant in need of said agent.
30. A method comprising administering the pharmaceutical composition of claim 24 to a plant in need of said agent.
31. A method comprising administering the pharmaceutical composition of claim 25 to a plant in need of said agent.
32. The method of any one of claims 26 to 28, wherein said individual has a fungal infection.

34. The method of any one of claims 29 to 31, wherein said administration is to protect said plant against disease.
- 5 35. The method of any one of claims 29 to 31, wherein said administration is to protect said plant against predation.

1/22



R=CH₃=vinblastine

R=CHO=vincristine

FIG. 1

2/22

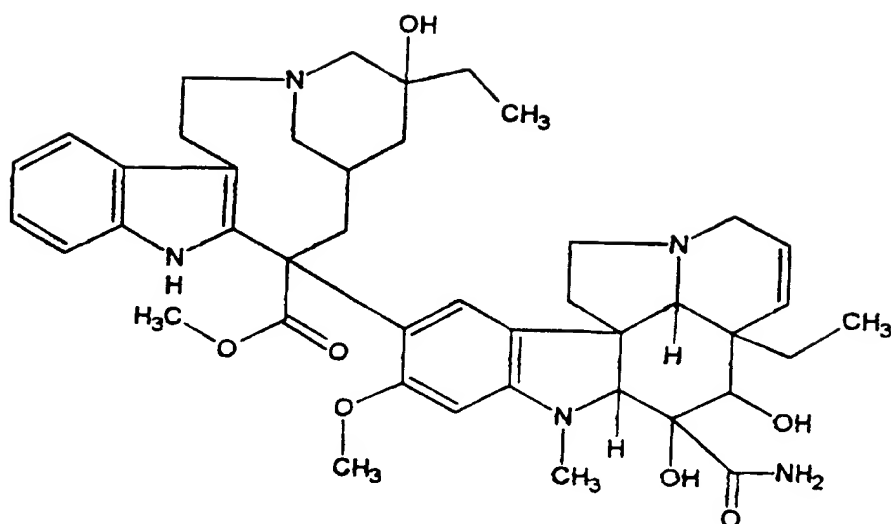


FIG. 2

3/22

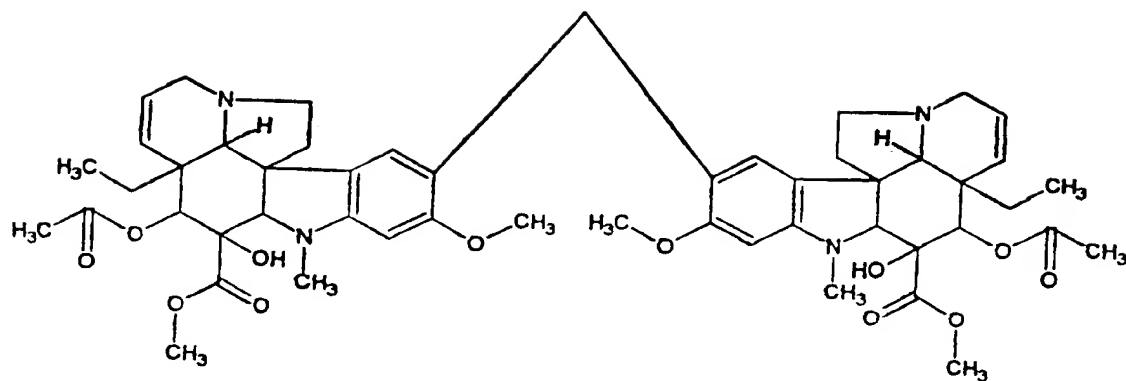


FIG. 3

4/22

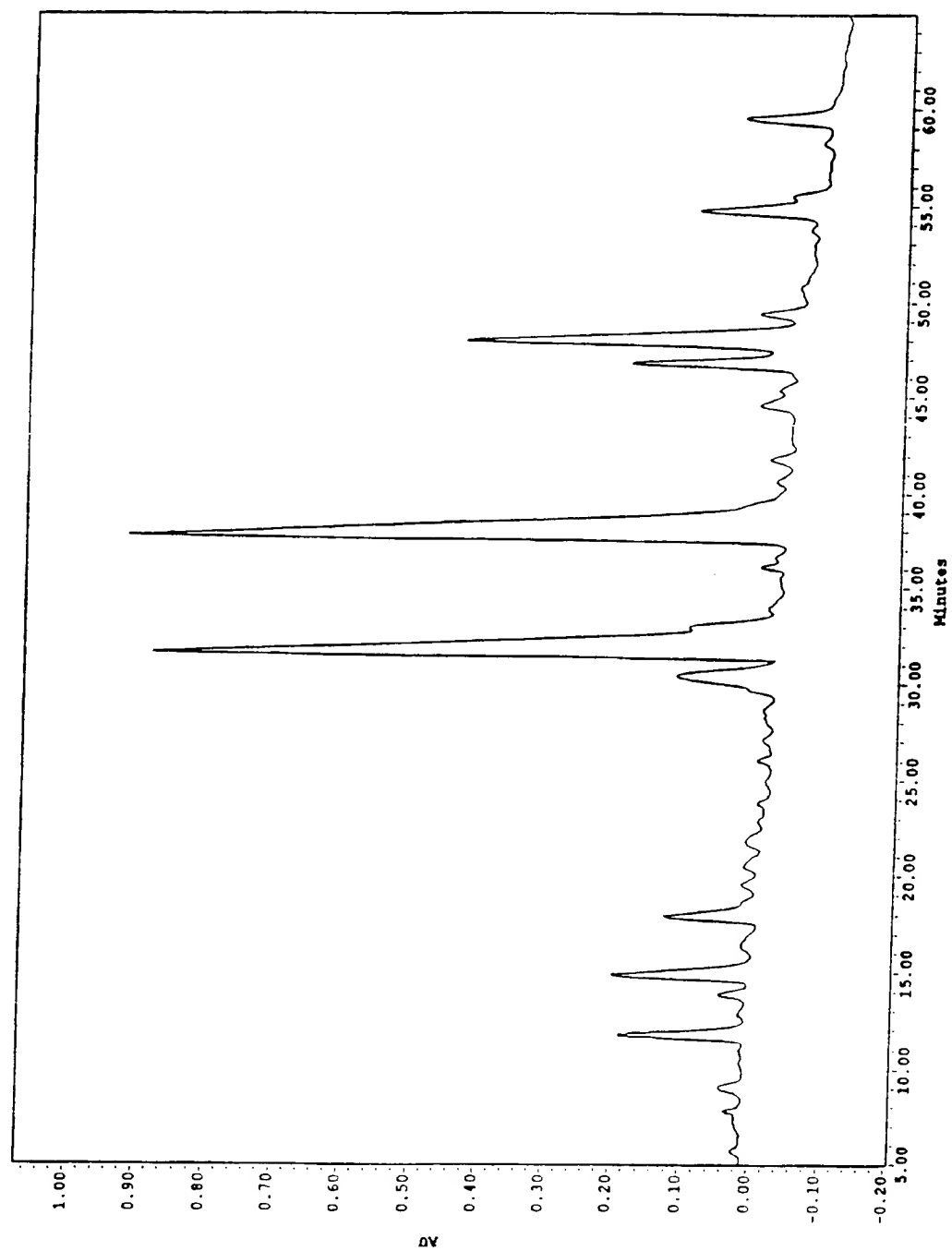


FIG. 4

5/22

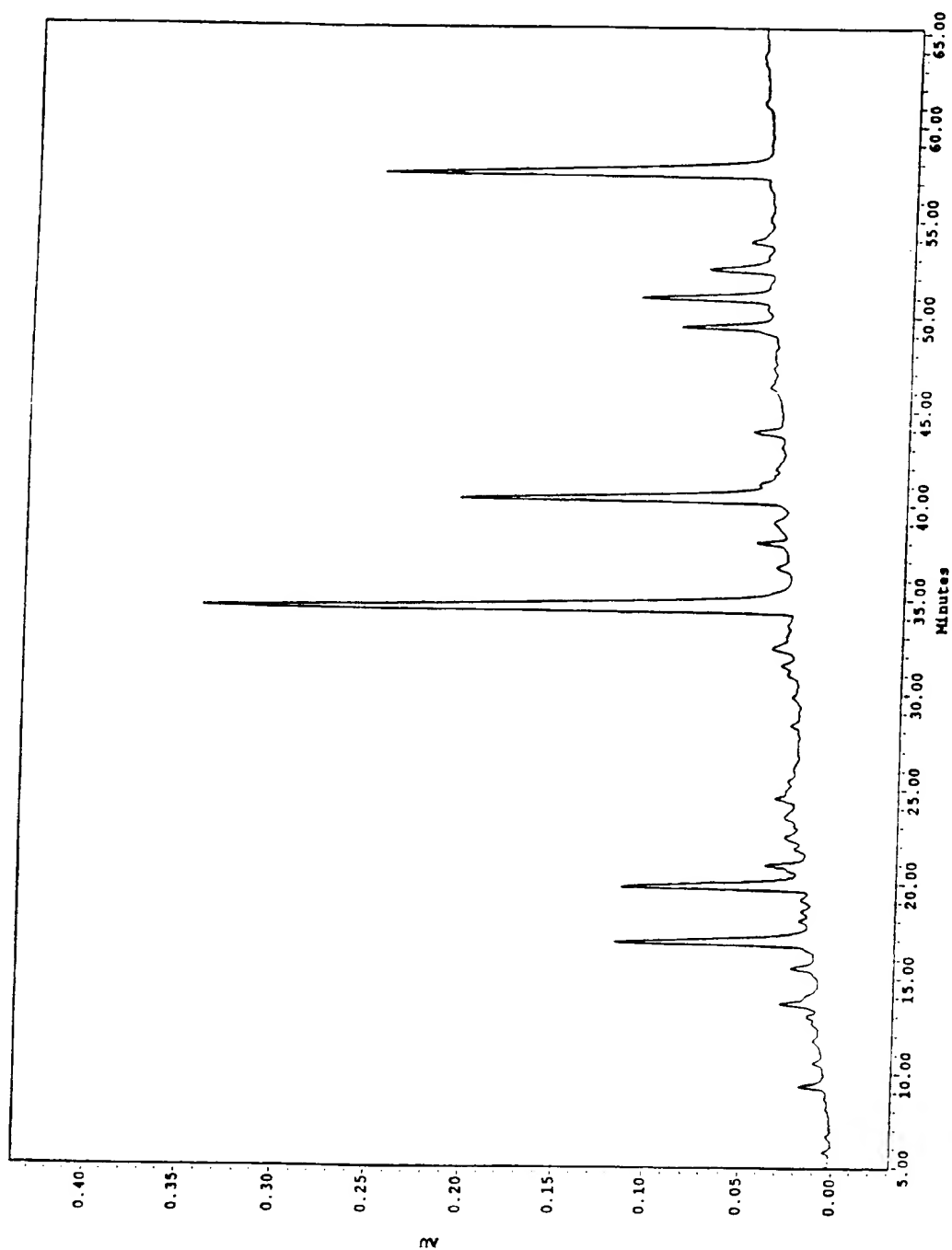


FIG. 5

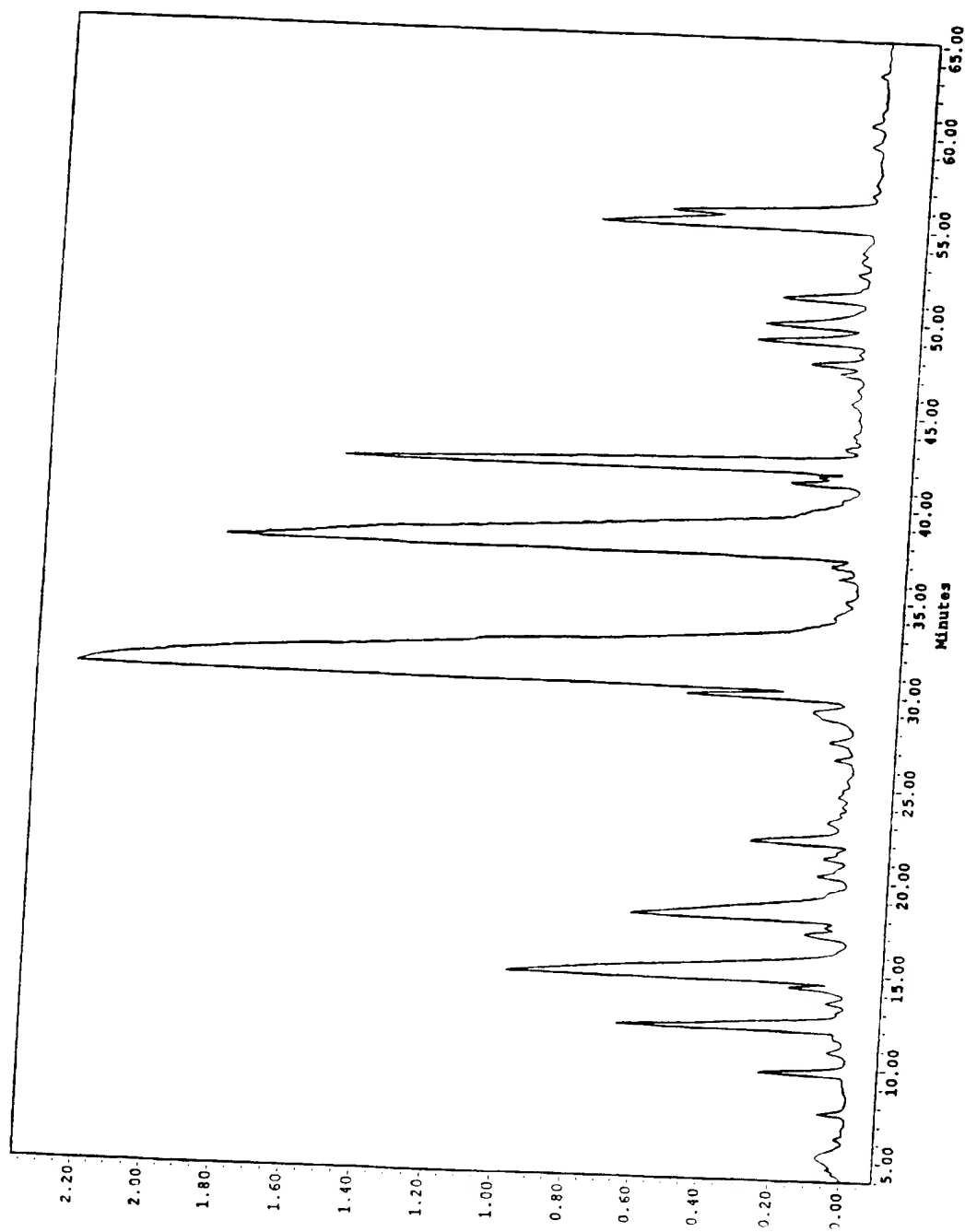


FIG. 6

7/22

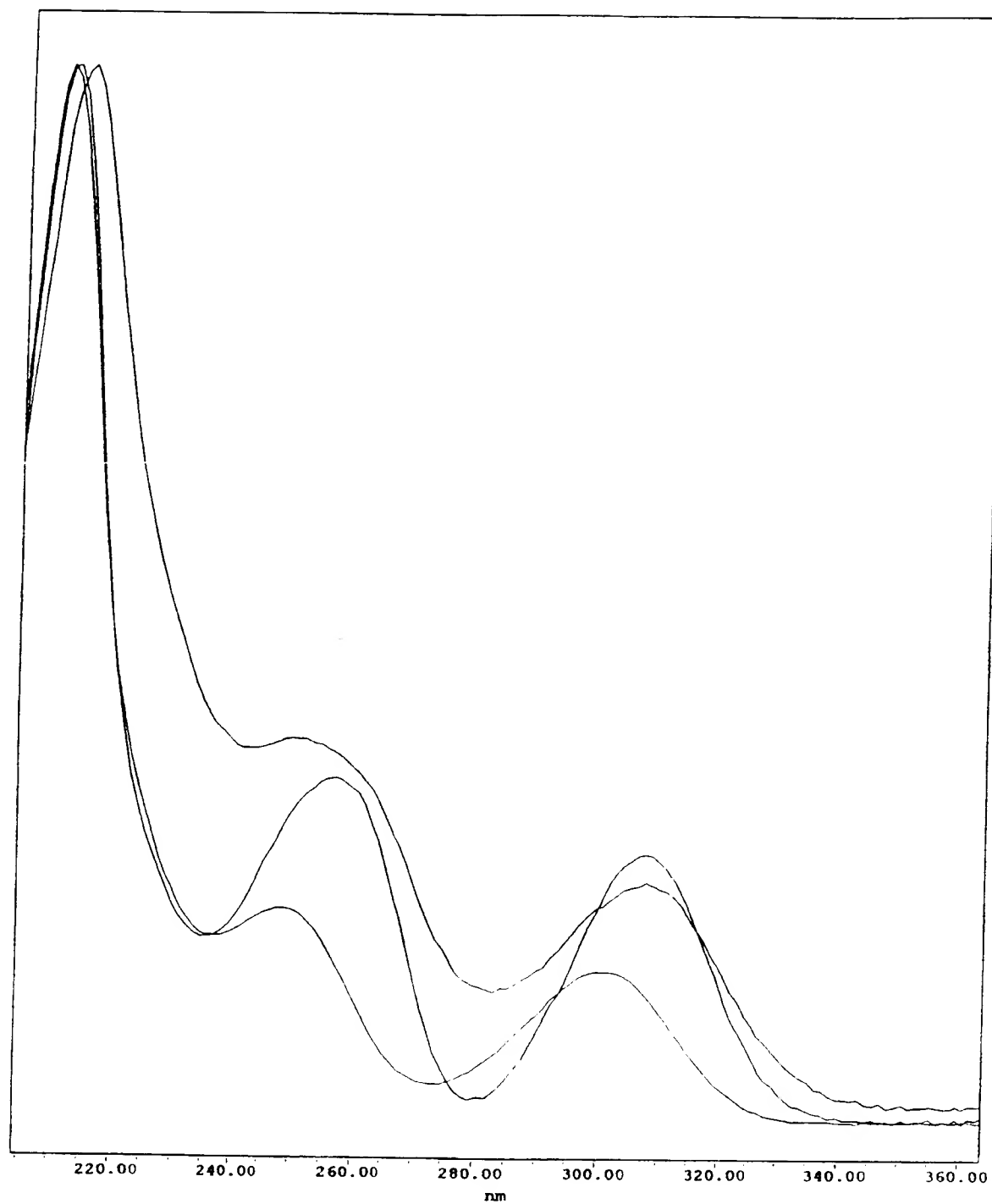


FIG. 7

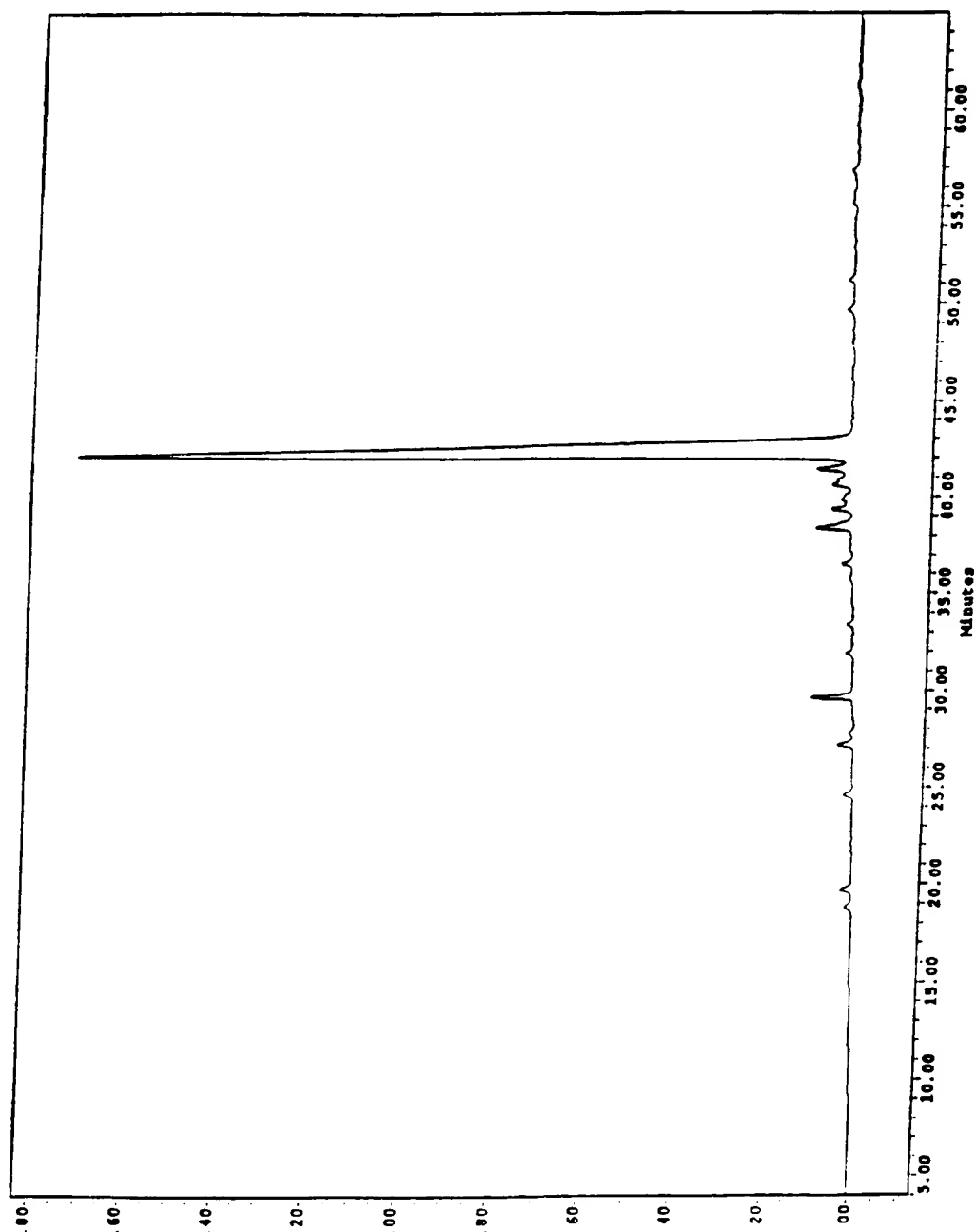


FIG. 8

9/22

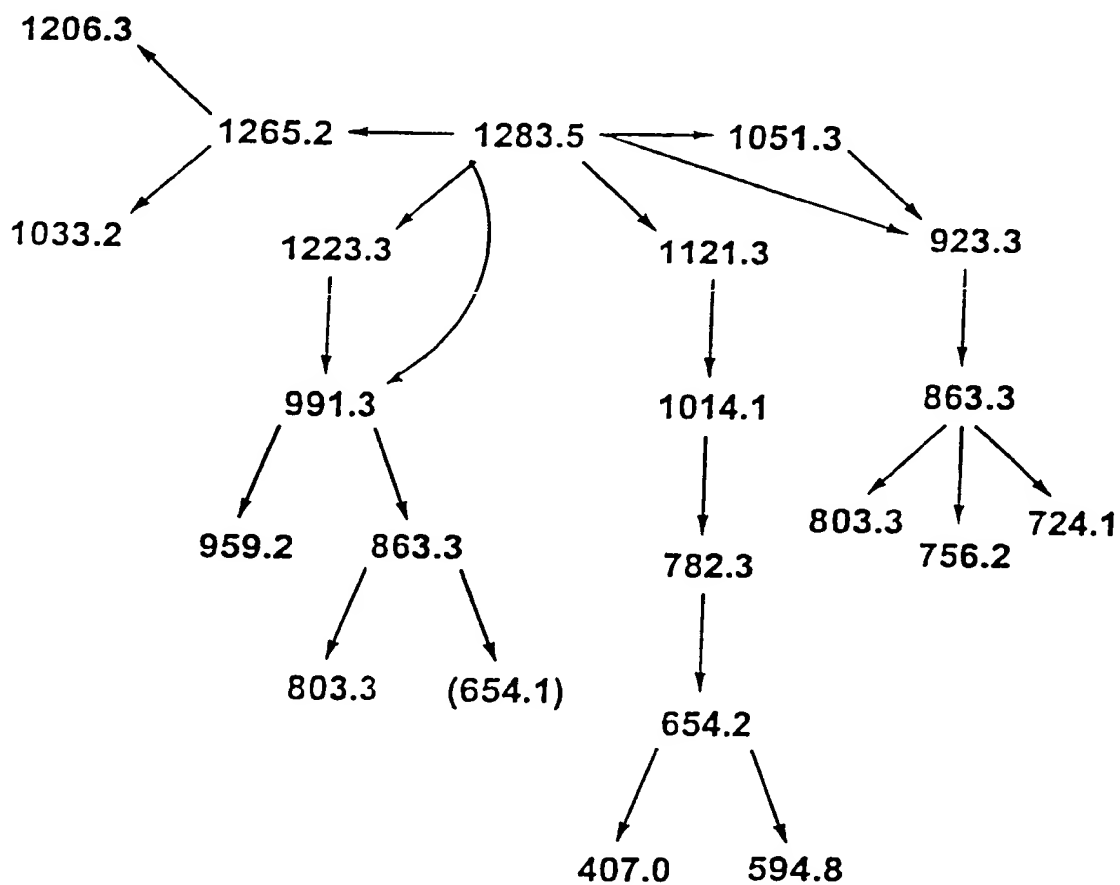


FIG. 9

10/22

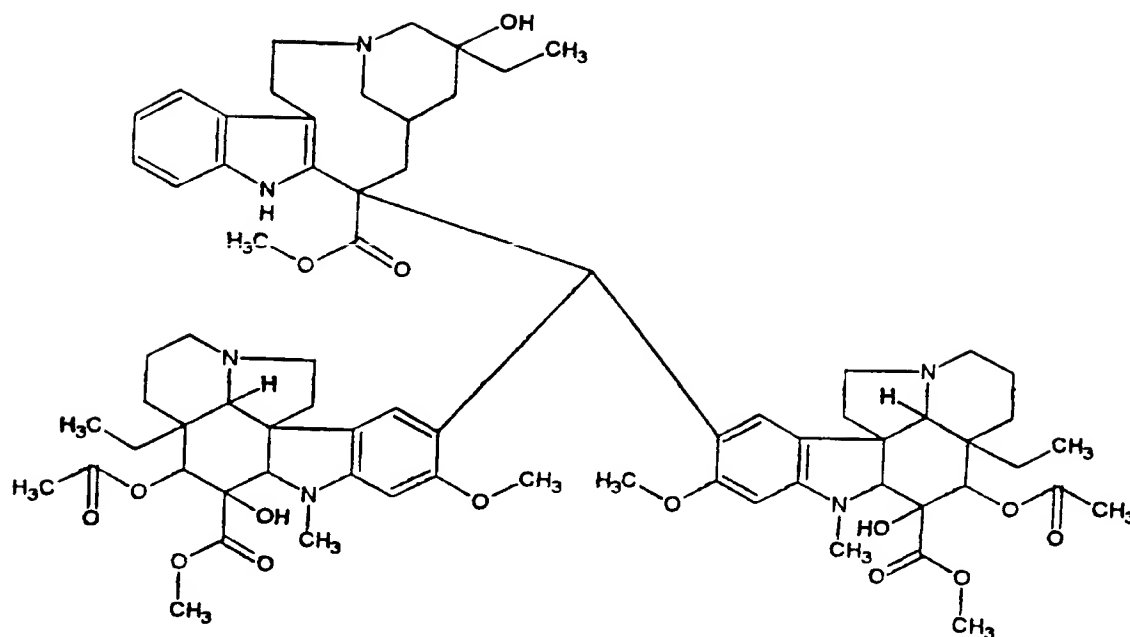


FIG. 10

11/22

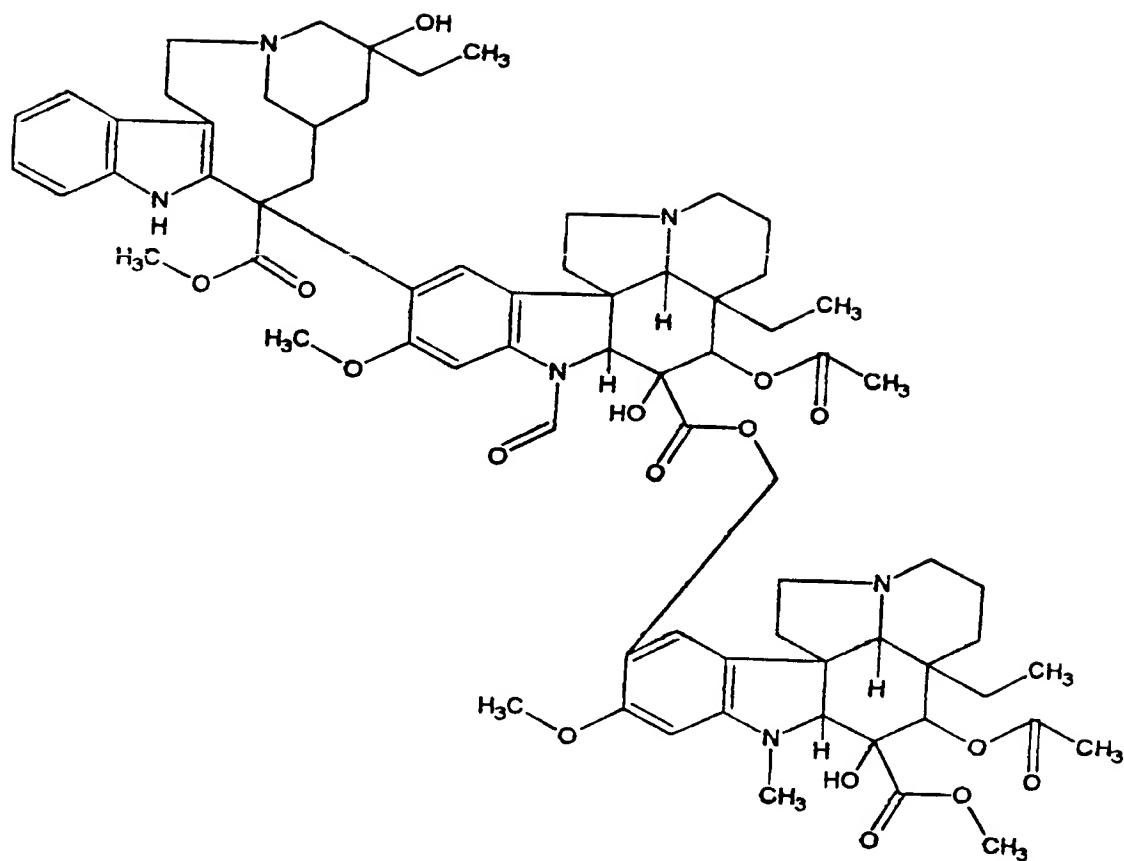


FIG. 11

12/22

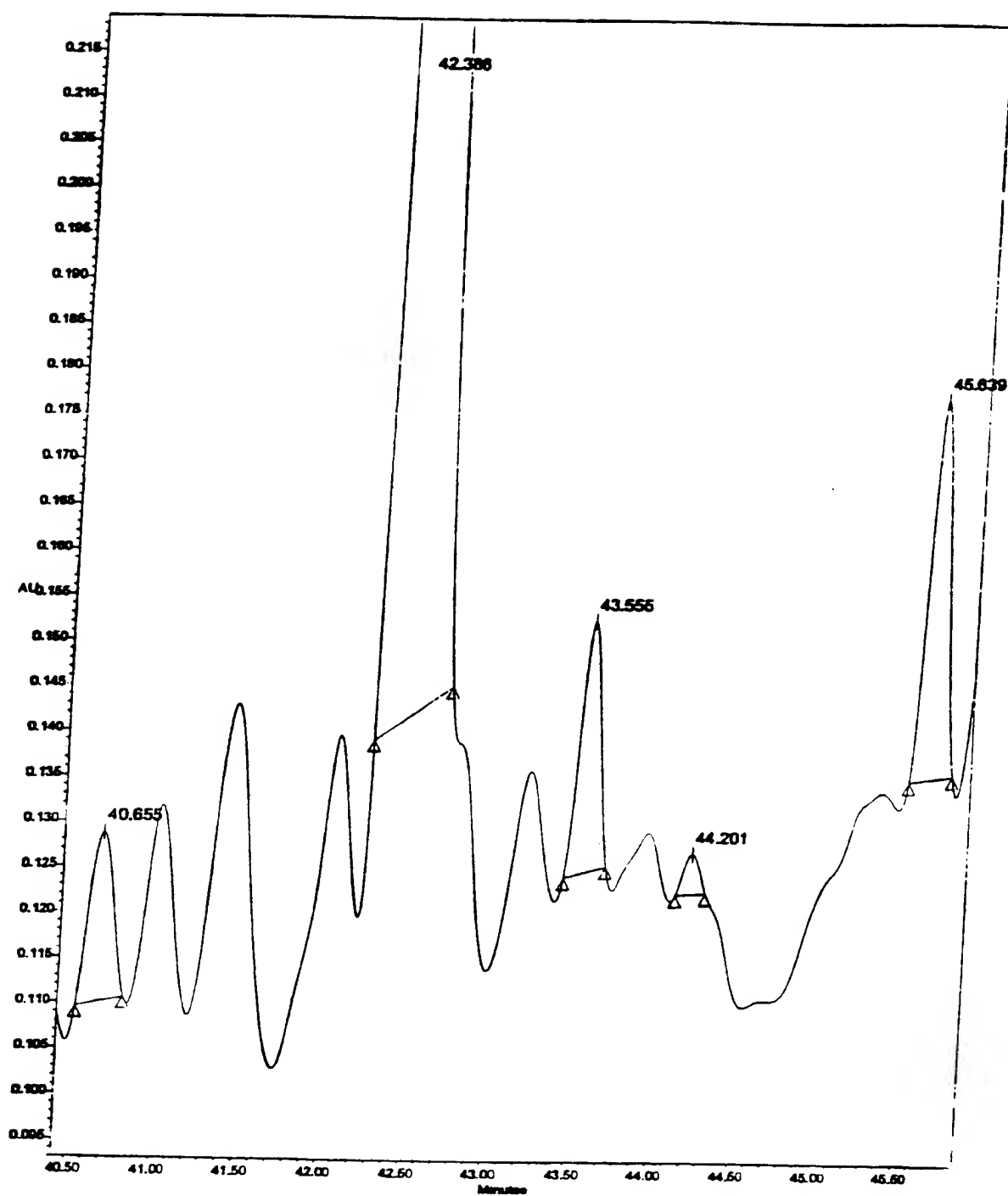


FIG. 12

13/22

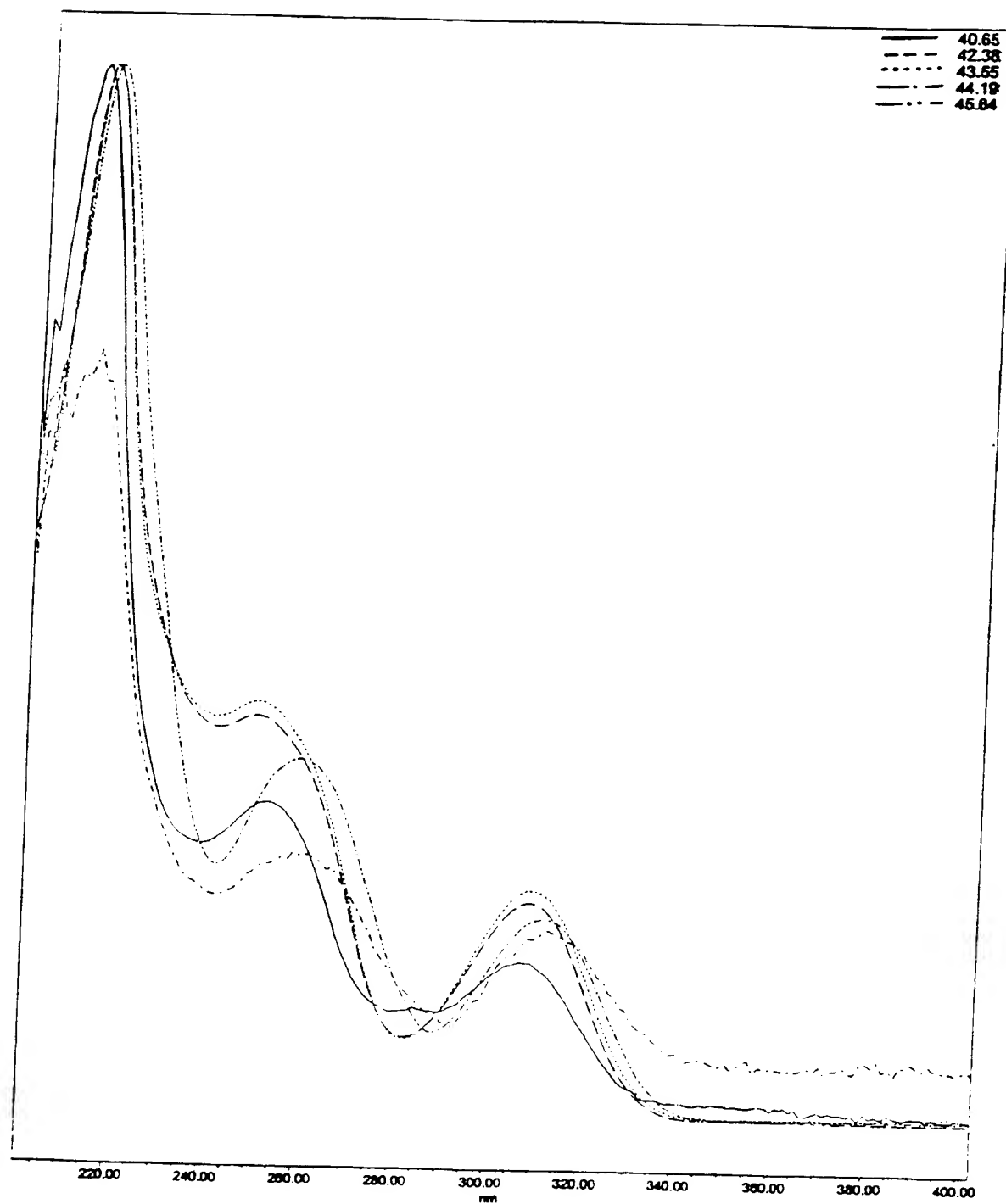


FIG. 13

14/22

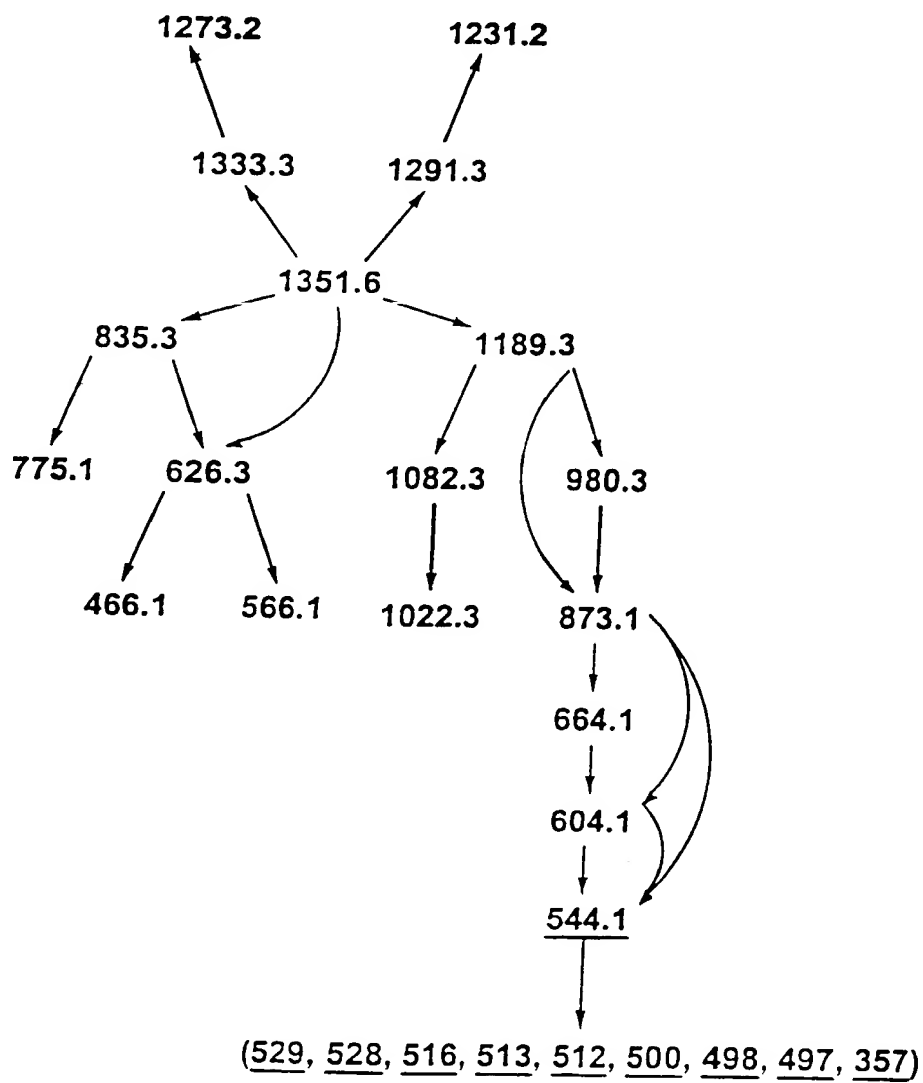


FIG. 14

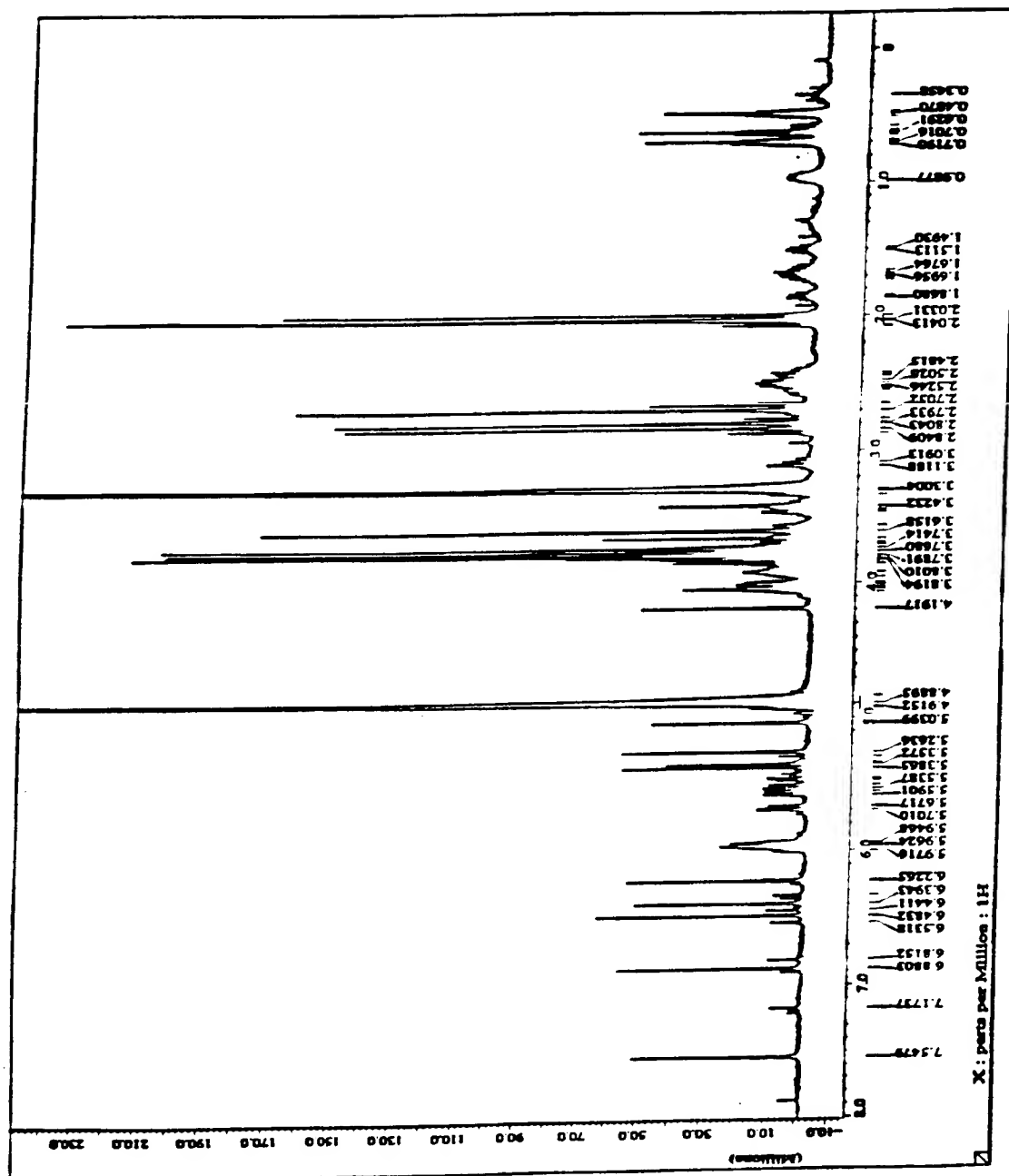


FIG. 15

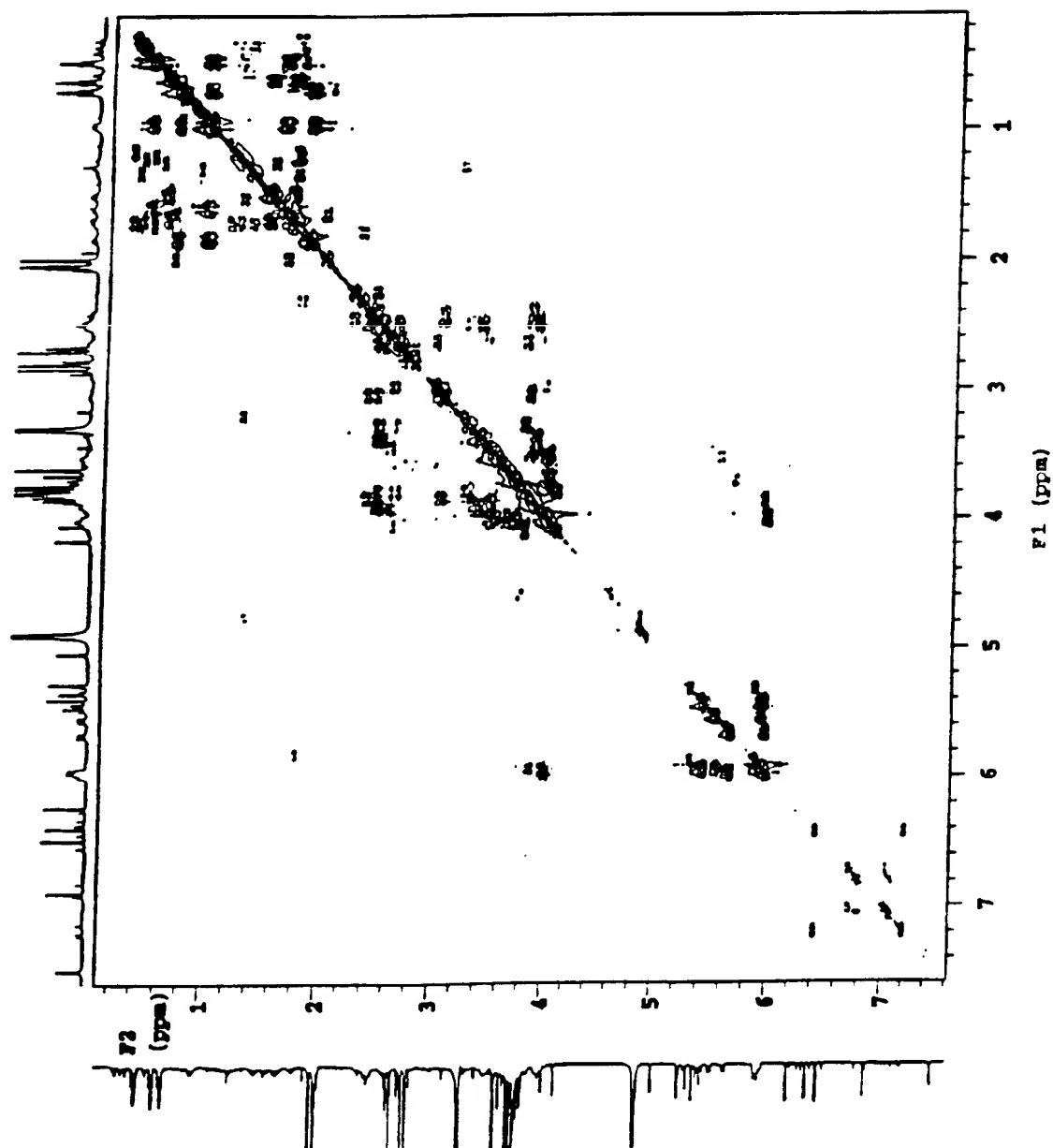


FIG. 16

17/22

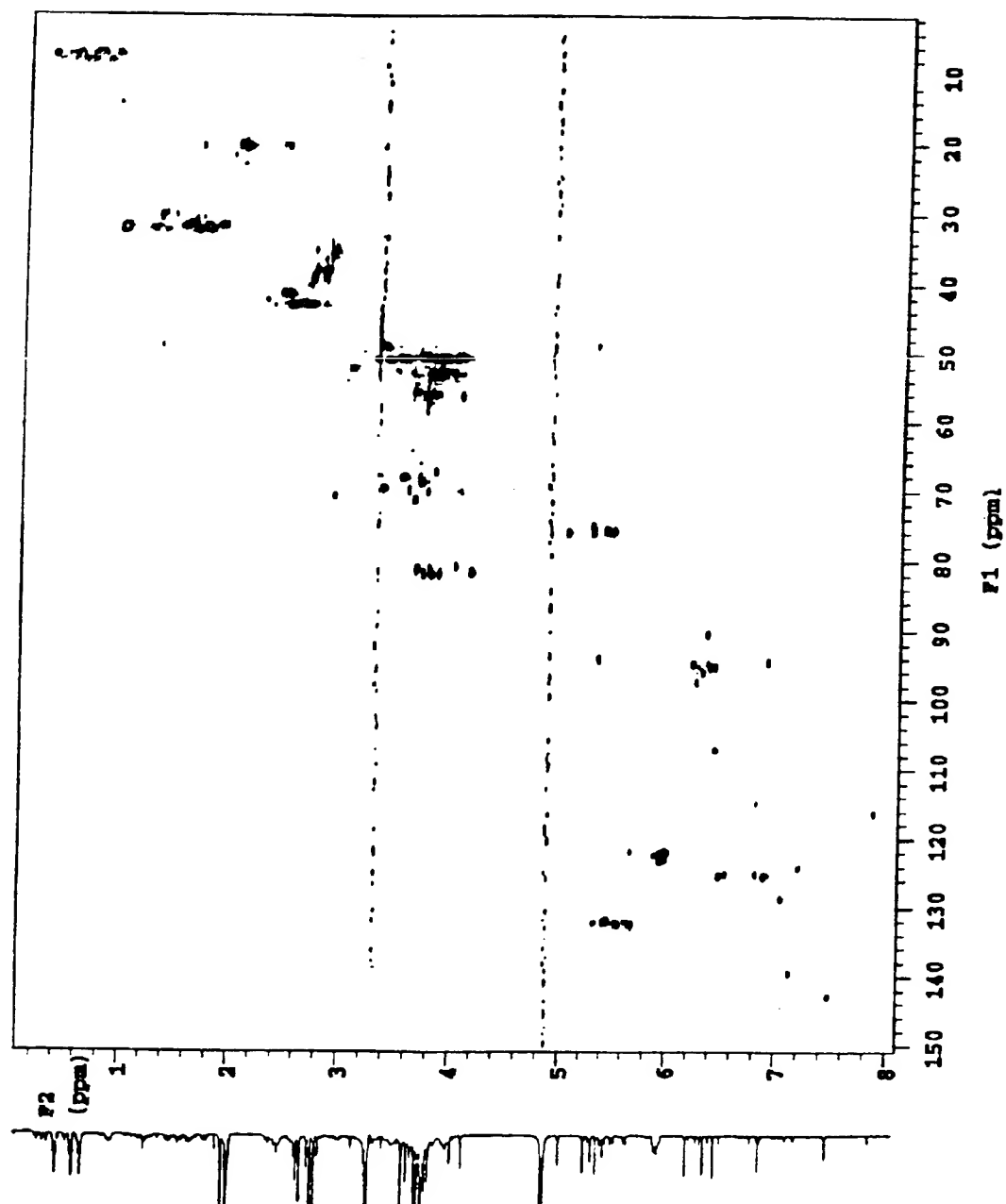


FIG. 17

18/22

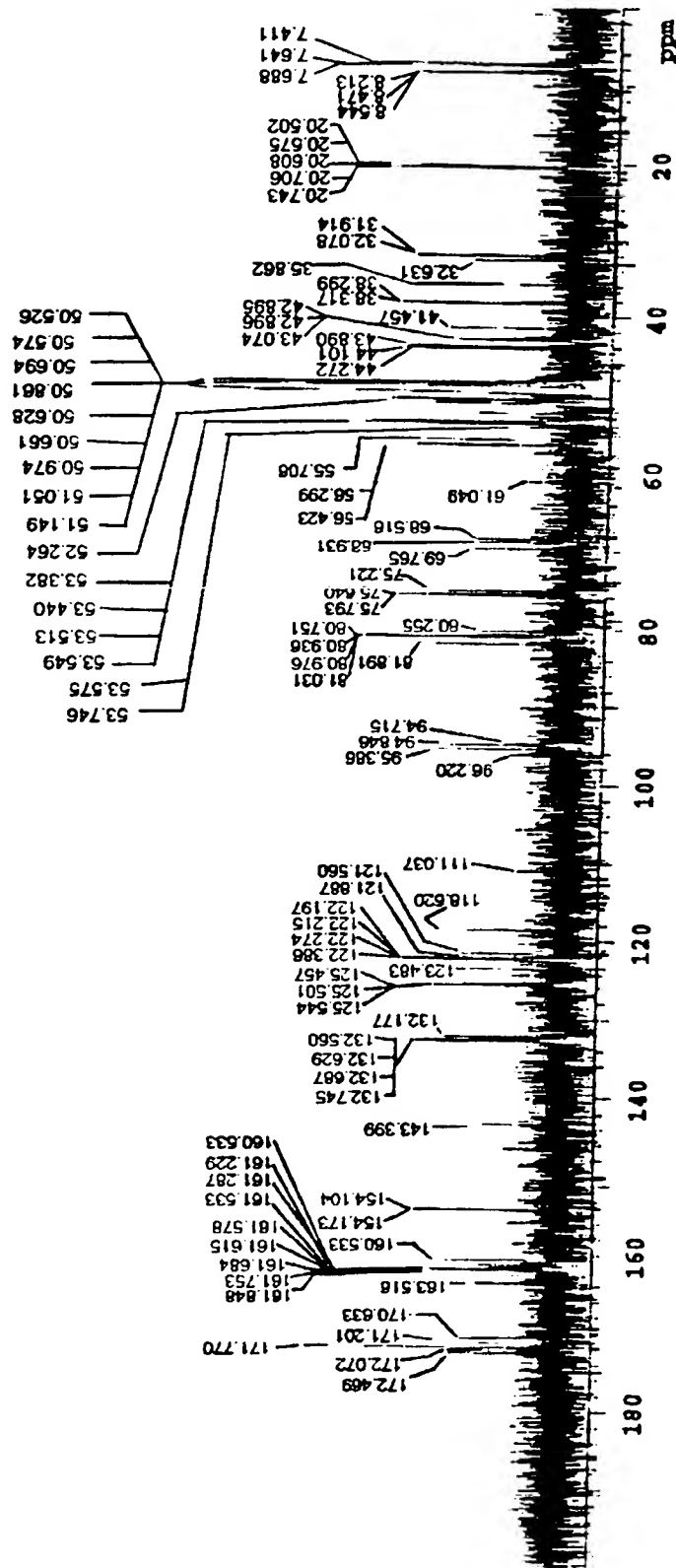


FIG. 18

19/22

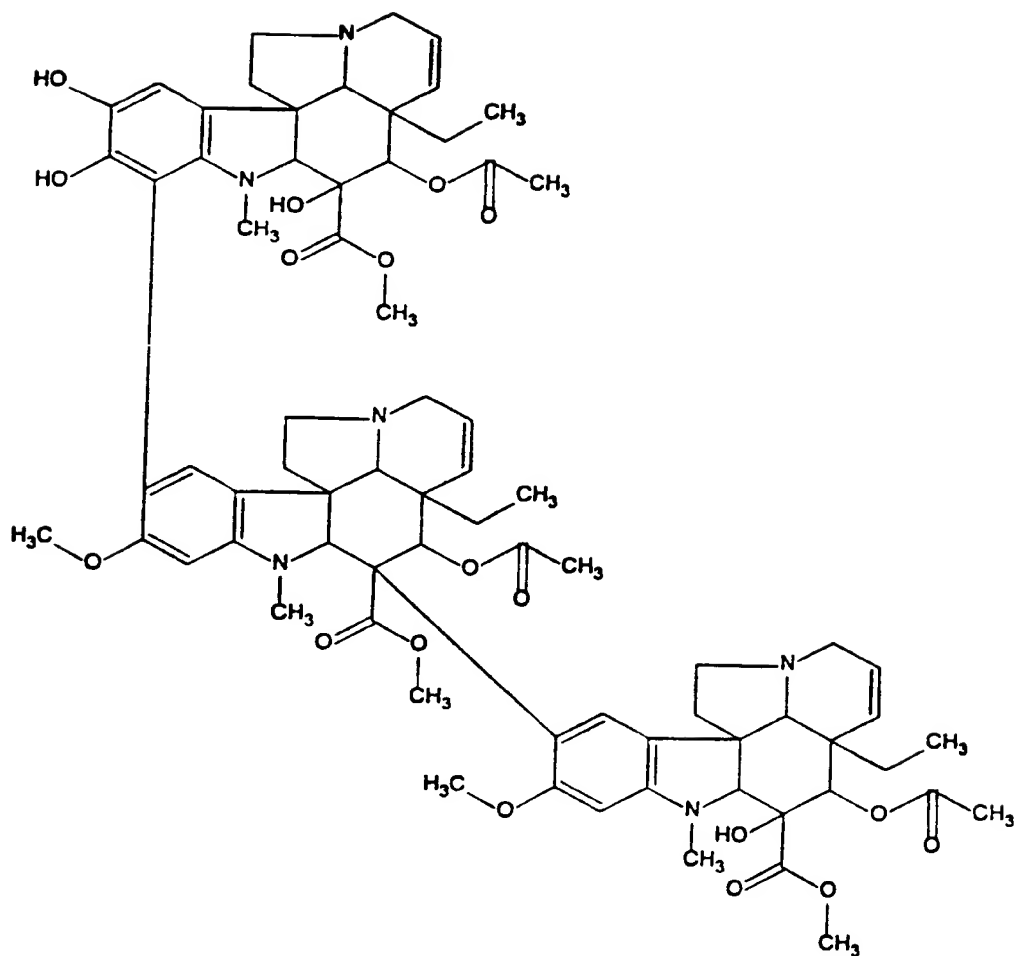


FIG. 19

20/22

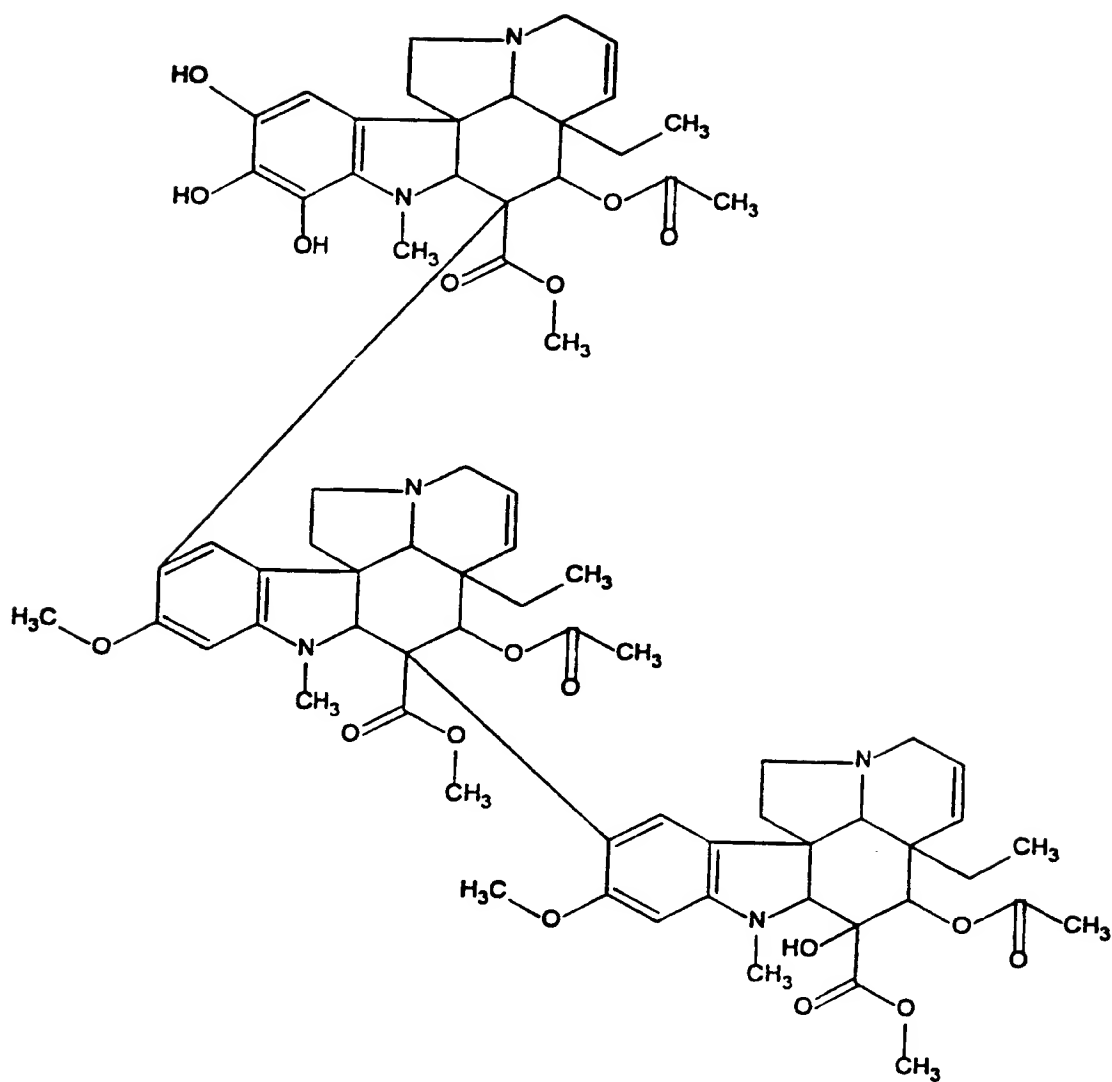


FIG. 20

21/22

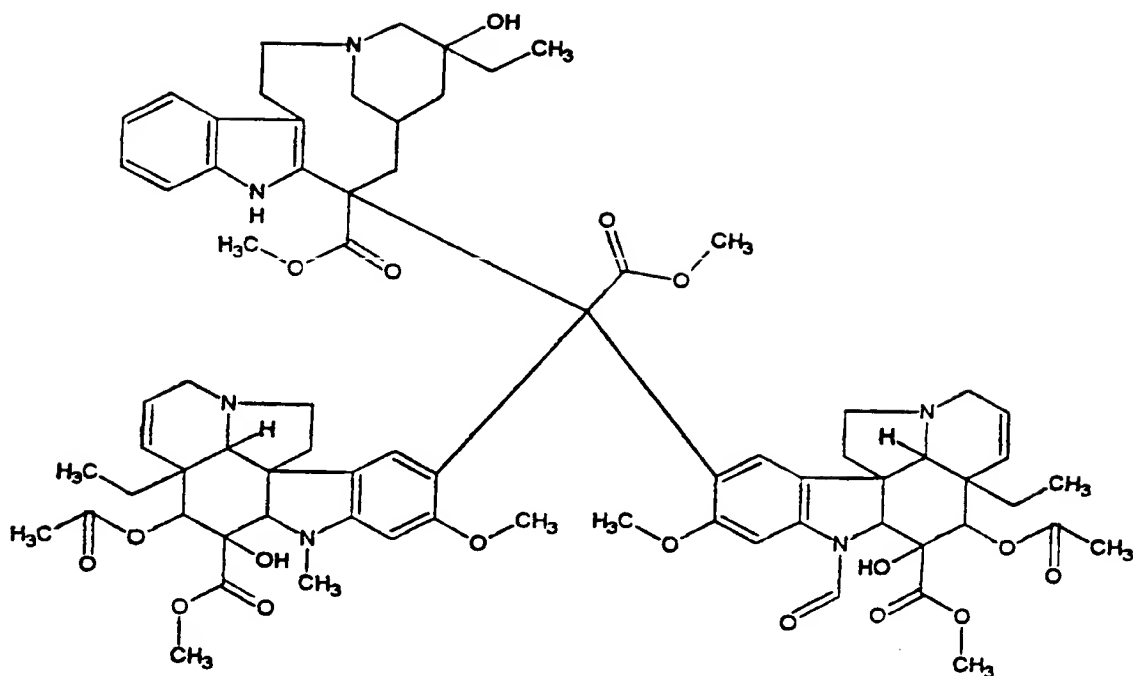


FIG. 21

22/22

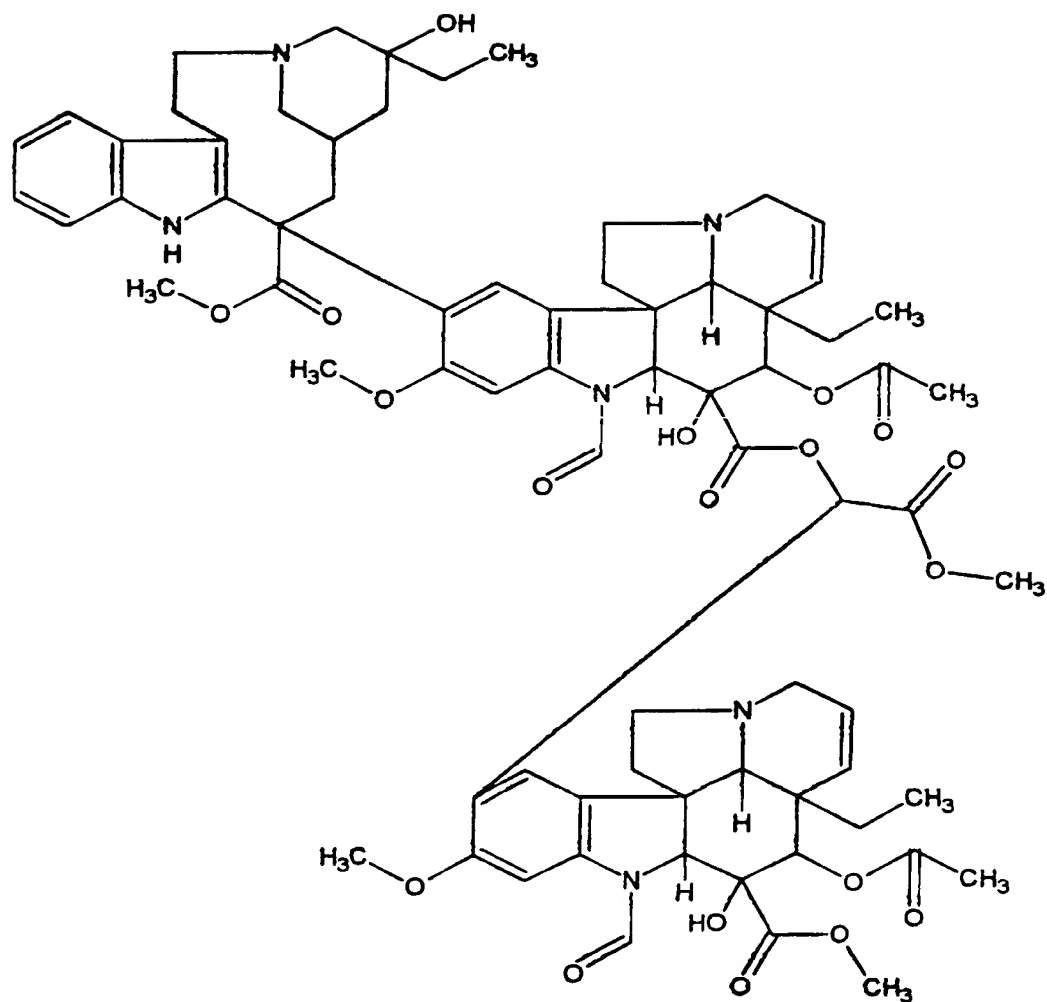


FIG. 22

INTERNATIONAL SEARCH REPORT

Intern. Application No.

PCT/US 99/17177

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07D519/04 C07D519/00 C07G5/00 A61K31/475

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07D C07G

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	R. A. CONRAD ET AL.: JOURNAL OF MEDICINAL CHEMISTRY, vol. 22, no. 4, 1979, pages 391-400, XP002121971 scheme II, compound 37; table I, compound 22; table III; page 397, right-hand column, lines 24-48; page 399, left-hand column, lines 12-70 ---	1
X	US 4 199 504 A (R. A. CONRAD ET AL.) 22 April 1980 (1980-04-22) example 1 ---	1
X	EP 0 233 101 A (IRE-CELLTARG S.A.) 19 August 1987 (1987-08-19) examples 2,3,5-11 --- -/--	1

☒ Further documents are listed in the continuation of box C☒ Patent family members are listed in annex

Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel: (+31-70) 340-2040, Tx: 31 651 epo.nl
Fax: (+31-70) 340-3016

Hass. C

INTERNATIONAL SEARCH REPORT

Intern Application No

PCT/US 99/17177

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 024 835 A (KSP S. BHUSHANA RAO ET AL.) 18 June 1991 (1991-06-18) cited in the application column 7, line 43 -column 8, line 21 ---	1
X	EP 0 222 722 A (OMNICHEM S. A.) 20 May 1987 (1987-05-20) examples 2,4,5 ---	1
X	US 5 030 620 A (J. A. A. HANNART ET AL.) 9 July 1991 (1991-07-09) cited in the application examples 11,12 ---	1
X	EP 0 041 935 A (OMNICHEM S. A.) 16 December 1981 (1981-12-16) examples 19-21 ---	1
X	EP 0 124 502 A (OMNICHEM S. A.) 7 November 1984 (1984-11-07) example 13 ---	1
X	US 4 203 898 A (G. J. CULLINAN ET AL.) 20 May 1980 (1980-05-20) cited in the application column 27, line 42 - line 49 ---	1
A	US 5 491 285 A (R. N. BOWMAN) 13 February 1996 (1996-02-13) cited in the application claim 1; example 12; table 9 ---	15
A	US 4 831 133 A (A. E. GOODBODY ET AL.) 16 May 1989 (1989-05-16) cited in the application ---	
A	US 4 172 077 A (K. JOVANOVIĆ ET AL.) 23 October 1979 (1979-10-23) cited in the application -----	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/17177

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 26-28, 32, 33
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 26-28, 32, 33
are directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition (Rule 39.1 iv PCT)
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were not paid by the applicant.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.

PCT/US 99/17177

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 4199504	A	22-04-1980	AU 4691379 A	22-11-1979
			BE 876155 A	12-11-1979
			CA 1114816 A	22-12-1981
			CA 1127153 A	06-07-1982
			CA 1121815 A	13-04-1982
			CS 214792 B	28-05-1982
			CS 214793 B	28-05-1982
			CS 214794 B	28-05-1982
			DD 143618 A	03-09-1980
			DD 147543 A	08-04-1981
			DD 150467 A	02-09-1981
			DK 197579 A	16-11-1979
			EP 0005620 A	28-11-1979
			EP 0033981 A	19-08-1981
			EP 0032756 A	29-07-1981
			ES 480560 A	01-04-1980
			ES 480561 A	01-04-1980
			ES 480562 A	01-04-1980
			FI 791519 A	16-11-1979
			FR 2435480 A	04-04-1980
			FR 2438052 A	30-04-1980
			GB 2022080 A	12-12-1979
			GR 73047 A	26-01-1984
			JP 54148798 A	21-11-1979
			LU 81254 A	10-09-1979
			PH 14999 A	22-03-1982
			PH 14907 A	29-01-1982
			PH 14904 A	29-01-1982
			PT 69589 A	01-06-1979
			RO 81682 A	29-04-1983
			ZA 7902271 A	31-12-1980
EP 233101	A	19-08-1987	FR 2592883 A	17-07-1987
			FR 2608158 A	17-06-1988
			CA 1335686 A	23-05-1995
			JP 1986205 C	08-11-1995
			JP 7005608 B	25-01-1995
			JP 62175486 A	01-08-1987
			US 4831038 A	16-05-1989
US 5024835	A	18-06-1991	FR 2626882 A	11-08-1989
			AT 108799 T	15-08-1994
			CA 1336119 A	27-06-1995
			DE 68916820 D	25-08-1994
			DE 68916820 T	09-03-1995
			EP 0328446 A	16-08-1989
			ES 2057157 T	16-10-1994
			JP 2152992 A	12-06-1990
			JP 2930965 B	09-08-1999
EP 222722	A	20-05-1987	LU 86157 A	26-06-1987
			DK 688451 A	14-06-1993
			DK 473286 A,B.	13-05-1987
			GR 862436 A	23-12-1986
			JP 62246599 A	27-10-1987

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.

PCT/US 99/17177

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 222722	A	NZ 218127 A	27-09-1989
		OA 8424 A	30-06-1988
US 5030620	A 09-07-1991	LU 86212 A	24-07-1987
		LU 86515 A	
		AU 592136 B	04-01-1990
		AU 6653786 A	18-06-1987
		DD 262998 A	21-12-1988
		DK 587986 A	17-06-1987
		EP 0232693 A	19-08-1987
		IL 80914 A	16-08-1991
		JP 62195387 A	28-08-1987
		NZ 218619 A	27-09-1989
		PT 83896 A,B	01-01-1987
		US 4828831 A	09-05-1989
EP 41935	A 16-12-1981	LU 82514 A	20-01-1982
		LU 83034 A	07-07-1982
		AT 390793 B	25-06-1990
		AT 253881 A	15-12-1989
		AT 9475 T	15-10-1984
		AU 541747 B	17-01-1985
		AU 7126281 A	17-12-1981
		BE 889136 A	09-12-1981
		CA 1196326 A	05-11-1985
		DD 160417 A	27-07-1983
		DE 3122479 A	16-06-1982
		DK 250181 A,B,	11-12-1981
		ES 502644 A	01-04-1982
		FR 2483926 A	11-12-1981
		GB 2078730 A	13-01-1982
		GR 74565 A	29-06-1984
		IE 51403 B	24-12-1986
		IT 1144601 B	29-10-1986
		JP 1586638 C	19-11-1990
		JP 2010836 B	09-03-1990
		JP 57028094 A	15-02-1982
		NL 8102802 A	04-01-1982
		NZ 197299 A	11-10-1985
		OA 6421 A	30-09-1981
		PT 73161 A,B	01-07-1981
		SE 8103594 A	11-12-1981
		US 4388305 A	14-06-1983
		US 4639456 A	27-01-1987
		ZA 8103781 A	30-06-1982
		PH 19031 A	06-12-1985
EP 124502	A 07-11-1984	LU 84784 A	28-11-1984
		LU 85161 A	12-09-1985
		AT 64396 T	15-06-1991
		AU 575819 B	11-08-1988
		GR 81965 A	11-11-1985
		GR 81790 A	12-12-1984
		JP 60034980 A	22-02-1985
		NZ 207992 A	30-04-1987

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.

PCT/US 99/17177

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 124502	A		OA 7711 A	30-06-1985
			PT 78508 A,B	01-05-1984
			US 4870162 A	26-09-1989
			DD 219771 A	13-03-1985
			IL 71642 A	05-11-1990
			PH 21394 A	15-10-1987

US 4203898	A	20-05-1980	US 4479957 A	30-10-1984

US 5491285	A	13-02-1996	AU 1683895 A	08-08-1995
			EP 0740504 A	06-11-1996
			JP 9508022 T	19-08-1997
			WO 9519698 A	27-07-1995

US 4831133	A	16-05-1989	CA 1262443 A	24-10-1989
			DE 3881819 A	22-07-1993
			DE 3881819 T	03-02-1994
			EP 0312609 A	26-04-1989
			FI 886053 A,B,	30-12-1988
			WO 8808425 A	03-11-1988
			JP 2550405 B	06-11-1996
			JP 1503236 T	02-11-1989

US 4172077	A	23-10-1979	AT 364469 B	27-10-1981
			AT 391578 A	15-03-1981
			AU 3664978 A	06-12-1979
			BE 867670 A	18-09-1978
			CA 1094552 A	27-01-1981
			CH 640861 A	31-01-1984
			DD 136139 A	20-06-1979
			DE 2823461 A	14-12-1978
			DK 239178 A,B,	01-12-1978
			FR 2393002 A	29-12-1978
			GB 1602633 A	11-11-1981
			IL 54761 A	13-09-1981
			IT 1096605 B	26-08-1985
			JP 54039100 A	24-03-1979
			MX 5237 E	11-05-1983
			NL 7805881 A	04-12-1978
			SE 437829 B	18-03-1985
			SE 7805900 A	01-12-1978
			YU 129378 A	31-10-1983

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference N1121-037.PCT	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/US 99/17177	International filing date (day/month/year) 29/07/1999	(Earliest) Priority Date (day/month/year) 31/07/1998
Applicant GOLDSMITH SEEDS, INC. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.



It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.



the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:



contained in the international application in written form.



filed together with the international application in computer readable form.



furnished subsequently to this Authority in written form.



furnished subsequently to this Authority in computer readable form.



the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.



the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2.



Certain claims were found unsearchable (See Box I).

3.



Unity of invention is lacking (see Box II).

4. With regard to the **title**,



the text is approved as submitted by the applicant.



the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,



the text is approved as submitted by the applicant.

has been established according to Rule 38.2(b) by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, request the Authority to

- a. The figure of the **drawings** to be published with the abstract is **Figure 1**.



as suggested by the applicant.



because the applicant failed to suggest a figure.



because this figure better characterizes the invention.

None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/17177

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 26-28,32,33
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 26-28,32,33 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition (Rule 39.1 iv PCT)
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US 99/17177

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07D519/04 C07D519/00 C07G5/00 A61K31/475

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07D C07G

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	R. A. CONRAD ET AL.: JOURNAL OF MEDICINAL CHEMISTRY, vol. 22, no. 4, 1979, pages 391-400, XP002121971 scheme II, compound 37; table I, compound 22; table III; page 397, right-hand column, lines 24-48; page 399, left-hand column, lines 12-70	1
X	US 4 199 504 A (R. A. CONRAD ET AL.) 22 April 1980 (1980-04-22) example 1	1
X	EP 0 233 101 A (IRE-CELLTARG S.A.) 19 August 1987 (1987-08-19) examples 2,3,5-11	1
	--- -/--	

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of mailing of the international search report

10 November 1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Hass, C

INTERNATIONAL SEARCH REPORT

 International Application No.
 PC/US 99/17177

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 024 835 A (KSP S. BHUSHANA RAO ET AL.) 18 June 1991 (1991-06-18) cited in the application column 7, line 43 - column 8, line 21	1
X	EP 0 222 722 A (OMNICHEM S. A.) 20 May 1987 (1987-05-20) examples 2,4,5	1
X	US 5 030 620 A (J. A. A. HANNART ET AL.) 9 July 1991 (1991-07-09) cited in the application examples 11,12	1
X	EP 0 041 935 A (OMNICHEM S. A.) 16 December 1981 (1981-12-16) examples 19-21	1
X	EP 0 124 502 A (OMNICHEM S. A.) 7 November 1984 (1984-11-07) example 13	1
X	US 4 203 898 A (G. J. CULLINAN ET AL.) 20 May 1980 (1980-05-20) cited in the application column 27, line 42 - line 49	1
A	US 5 491 285 A (R. N. BOWMAN) 13 February 1996 (1996-02-13) cited in the application claim 1; example 12; table 9	15
A	US 4 831 133 A (A. E. GOODBODY ET AL.) 16 May 1989 (1989-05-16) cited in the application	
A	US 4 172 077 A (K. JOVANOVIĆ ET AL.) 23 October 1979 (1979-10-23) cited in the application	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/17177

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4199504 A	22-04-1980	AU 4691379 A	22-11-1979
		BE 876155 A	12-11-1979
		CA 1114816 A	22-12-1981
		CA 1127153 A	06-07-1982
		CA 1121815 A	13-04-1982
		CS 214792 B	28-05-1982
		CS 214793 B	28-05-1982
		CS 214794 B	28-05-1982
		DD 143618 A	03-09-1980
		DD 147543 A	08-04-1981
		DD 150467 A	02-09-1981
		DK 197579 A	16-11-1979
		EP 0005620 A	28-11-1979
		EP 0033981 A	19-08-1981
		EP 0032756 A	29-07-1981
		ES 480560 A	01-04-1980
		ES 480561 A	01-04-1980
		ES 480562 A	01-04-1980
		FI 791519 A	16-11-1979
		FR 2435480 A	04-04-1980
		FR 2438052 A	30-04-1980
		GB 2022080 A	12-12-1979
		GR 73047 A	26-01-1984
		JP 54148798 A	21-11-1979
		LU 81254 A	10-09-1979
		PH 14999 A	22-03-1982
		PH 14907 A	29-01-1982
		PH 14904 A	29-01-1982
		PT 69589 A	01-06-1979
		RO 81682 A	29-04-1983
		ZA 7902271 A	31-12-1980
EP 233101 A	19-08-1987	FR 2592883 A	17-07-1987
		FR 2608158 A	17-06-1988
		CA 1335686 A	23-05-1995
		JP 1986205 C	08-11-1995
		JP 7005608 B	25-01-1995
		JP 62175486 A	01-08-1987
		US 4831038 A	16-05-1989
US 5024835 A	18-06-1991	FR 2626882 A	11-08-1989
		AT 108799 T	15-08-1994
		CA 1336119 A	27-06-1995
		DE 68916820 D	25-08-1994
		DE 68916820 T	09-03-1995
		EP 0328446 A	16-08-1989
		ES 2057157 T	16-10-1994
		JP 2152992 A	12-06-1990
		JP 2930965 B	09-08-1999
EP 222722 A	20-05-1987	LU 86157 A	26-06-1987
		AT 89567 T	15-06-1993
		DE 3206186 A	04-04-1988
		DE 340186 A	04-04-1988
		DE 3688451 A	24-06-1993
		DK 473286 A, B,	13-05-1987
		GR 862436 A	23-12-1986
		JP 62246599 A	27-10-1987

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/17177

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 222722	A		NZ 218127 A OA 8424 A	27-09-1989 30-06-1988
US 5030620	A	09-07-1991	LU 86212 A LU 86515 A AU 592136 B AU 6653786 A DD 262998 A DK 587986 A EP 0232693 A IL 80914 A JP 62195387 A NZ 218619 A PT 83896 A, B US 4828831 A	24-07-1987 04-01-1990 18-06-1987 21-12-1988 17-06-1987 19-08-1987 16-08-1991 28-08-1987 27-09-1989 01-01-1987 09-05-1989
EP 41935	A	16-12-1981	LU 82514 A LU 83034 A AT 390793 B AT 253881 A AT 9475 T AU 541747 B AU 7126281 A BE 889136 A CA 1196326 A DD 160417 A DE 3122479 A DK 250181 A, B, ES 502644 A FR 2483926 A GB 2078730 A GR 74565 A IE 51403 B IT 1144601 B JP 1586638 C JP 2010836 B JP 57028094 A NL 8102802 A NZ 197299 A OA 6421 A PT 73161 A, B SE 8103594 A US 4388305 A US 4639456 A ZA 8103781 A PH 19031 A	20-01-1982 07-07-1982 25-06-1990 15-12-1989 15-10-1984 17-01-1985 17-12-1981 09-12-1981 05-11-1985 27-07-1983 16-06-1982 11-12-1981 01-04-1982 11-12-1981 13-01-1982 29-06-1984 24-12-1986 29-10-1986 19-11-1990 09-03-1990 15-02-1982 04-01-1982 11-10-1985 30-09-1981 01-07-1981 11-12-1981 14-06-1983 27-01-1987 30-06-1982 06-12-1985
EP 124502	A	07-11-1984	LU 84784 A LU 85161 A AT 64396 T AU 575819 B AU 2743484 A AU 234800 AU 20784 ES 531965 A GR 81790 A JP 60034980 A NZ 207992 A	28-11-1984 12-09-1985 15-06-1991 11-08-1988 01-11-1984 04-1988 04-1984 01-11-1985 12-12-1984 22-02-1985 30-04-1987

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/17177

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 124502 A		OA 7711 A	30-06-1985
		PT 78508 A,B	01-05-1984
		US 4870162 A	26-09-1989
		DD 219771 A	13-03-1985
		IL 71642 A	05-11-1990
		PH 21394 A	15-10-1987
US 4203898 A	20-05-1980	US 4479957 A	30-10-1984
US 5491285 A	13-02-1996	AU 1683895 A	08-08-1995
		EP 0740504 A	06-11-1996
		JP 9508022 T	19-08-1997
		WO 9519698 A	27-07-1995
US 4831133 A	16-05-1989	CA 1262443 A	24-10-1989
		DE 3881819 A	22-07-1993
		DE 3881819 T	03-02-1994
		EP 0312609 A	26-04-1989
		FI 886053 A,B,	30-12-1988
		WO 8808425 A	03-11-1988
		JP 2550405 B	06-11-1996
		JP 1503236 T	02-11-1989
US 4172077 A	23-10-1979	AT 364469 B	27-10-1981
		AT 391578 A	15-03-1981
		AU 3664978 A	06-12-1979
		BE 867670 A	18-09-1978
		CA 1094552 A	27-01-1981
		CH 640861 A	31-01-1984
		DD 136139 A	20-06-1979
		DE 2823461 A	14-12-1978
		DK 239178 A,B,	01-12-1978
		FR 2393002 A	29-12-1978
		GB 1602633 A	11-11-1981
		IL 54761 A	13-09-1981
		IT 1096605 B	26-08-1985
		JP 54039100 A	24-03-1979
		MX 5237 E	11-05-1983
		NL 7805881 A	04-12-1978
		SE 437829 B	18-03-1985
		SE 7805900 A	01-12-1978
		YU 129378 A	31-10-1983